Calmodulin-regulated Binding of the 90-kDa Heat Shock Protein to Actin Filaments

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We have found that the 90-kDa heat shock protein (HSP90) prepared from a mouse lymphoma exists in homodimeric form under physiological conditions and has the ability to bind to F-actin (Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H., and Yahara, I. (1986) Proc. Natl. Acad. Sci. U. S. A., in press). Here we show that calmodulin regulates the binding of HSP90 to F-actin in a Ca2+-dependent manner. The binding of HSP90 to F-actin occurred optimally under physiological solution conditions, i.e. in 2 mM MgCl2 + 100 mM KCl. The binding was saturable in a molar ratio of about 1 HSP90 (dimer) to 10 actins. HSP90 was dissociated from F-actin by the binding of tropomyosin to F-actin. Calmodulin was found to inhibit the binding of HSP90 to F-actin in a Ca2+-dependent manner. Moreover, the equilibrium gel filtration demonstrated that calmodulin binds to HSP90 in the presence of Ca2+, but not in the absence of Ca2+. These data indicate that HSP90 complexed with Ca2+-calmodulin is unable to bind to F-actin. Ca2+-dependent interaction of HSP90 with calmodulin as well as calmodulin-regulated binding of HSP90 to F-actin revealed here may provide new insight into the function of HSP90 and the regulation of actin structure in cells.

A wide range of cells and organisms synthesizes a limited number of specific proteins, called heat shock proteins, when exposed to elevated temperatures or other environmental stresses (1, 2). Species of heat shock proteins and their amino acid sequences have been conserved throughout evolution (3, 4). Moreover, at least some heat shock proteins are synthesized constitutively (5–7), and several heat shock proteins have recently been identified as previously known proteins which play important roles in cellular functions (8–10). These significant proteins such as pp60src or steroid hormone receptors associate transiently with pp60src, ppl40src, and gc94src in avian cells (11–14). More recently, it has been suggested that HSP90 may be associated with the steroid hormone receptor and that upon activation or transformation the receptor dissociates from HSP90 (15–18). It is possible that in these cases HSP90 functions as a carrier protein. Most recently, we have found that HSP90 is an actin-binding protein and is localized in ruffling membranes of cultured cells (19). Thus, it is tempting to speculate that HSP90 transports biologically significant proteins such as pp60src or steroid hormone receptor through interaction with actin. In this study, evidence is presented that calmodulin is capable of binding to HSP90 in a Ca2+-dependent manner and therefore regulates the interaction of HSP90 with actin.

MATERIALS AND METHODS

Purification of Proteins—Calmodulin was purified from porcine brains described previously (20). Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (21) and further purified by gel filtration on Sephadex G-100 equilibrated with a buffer solution containing 0.1 mM CaCl2, 0.2 mM ATP, 0.1 mM dithiothreitol, 0.01% NaN3, and 2 mM HEPES, pH 7.8. Actin concentration was determined by UV absorption measurement based on A254 = 6.8. HSP90 was purified from a mouse lymphoma cell line, L5178Y, by a new method that involved sequential chromatography on DEAE-cellulose and hydroxylapatite. The detailed description of this new purification procedure will be described elsewhere.2 Tropomyosin purified from bovine gastric mucosa smooth muscle (22) was kindly given by Dr. S. Maekawa (University of Tokyo).

Assay of Binding of HSP90 and Tropomyosin to F-Actin—This was performed by the pelleting assay. Actin was incubated either in the presence or absence of HSP90 or tropomyosin at 25 °C for 1–2 h in the standard buffer solution consisting of 2 mM MgCl2, 100 mM KCl, 0.7 mM EGTA, 0.04 mM ATP, and 30 mM PIPES, pH 6.7. An aliquot of each solution then was centrifuged at 100,000 × g for 2 h. The resulting supernatant and pellet fractions were electrophoresed on SDS-polyacrylamide gels (7.5 or 10% acrylamide). Gels were stained with Coomassie Blue, and the intensity of the stained band was determined by scanning the gels with a densitometer.

Equilibrium Gel Filtration—A mixture (0.3 ml) of calmodulin (0.04 mg/ml) and HSP90 (1.95 mg/ml) either with or without Ca2+ was passed through a Sephadex G-100 column (0.9 × 15-cm) equilibrated with a buffer solution (30 mM PIPES, 2 mM MgCl2, 100 mM KCl, pH 6.7) containing the same concentration of calmodulin in the presence (1 mM CaCl2 or 0.7 mM EGTA) or absence (0.7 mM EGTA) of Ca2+ at 25 °C. Each fraction was analyzed by SDSPolyacrylamide gel electrophoresis, and the concentration of calmodulin was determined by densitometry as described above.

Other Procedures—SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere (23). Protein concentration was determined following Lowry et al. (24) using bovine serum albumin as a standard. Actin polymerization process was measured by monitoring the absorbance change at 237 nm with a Gilford 260 spectrophotometer in a temperature-controlled cuvette chamber (25, 26). When Ca2+-EGTA buffer was used, free Ca2+ concentrations were calculated from the apparent association of EGTA with Ca2+ (Kapp) = 5 × 105 M−1 at pH 6.7 (20).

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The abbreviations used are: HSP90, 90-kDa heat shock protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenediaminetetraacetic acid; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate.

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RESULTS

When actin was polymerized in the presence or absence of HSP90, there was no substantial difference in the extent of actin polymerization, i.e. the amount of F-actin formed (Fig. 1). It was also found that HSP90 did not affect actin polymerization kinetics which were measured by monitoring the absorbance change at 237 nm (data not shown). However, it was clearly shown that HSP90 cosediments with F-actin (Fig. 1), as in our previous report (19). The amount of bound HSP90 increased with increasing KCl concentration up to 100 mM and then decreased gradually. The binding also depended on the concentration of Mg\(^{2+}\). The maximal binding was observed at \(\pm 2\) mM Mg\(^{2+}\) (up to 5 mM), and the binding was weakened by decreasing the Mg\(^{2+}\) concentration lower than 2 mM (data not shown). When increasing concentrations of HSP90 were reacted with F-actin, the amount of bound HSP90 increased and seemed to attain a plateau level (Fig. 2A), indicating that the binding is saturable. A reciprocal plot of the data was linear and gave a dissociation constant \(K_D\) and a binding capacity at saturation (Fig. 2B). In 2 mM Mg\(^{2+}\) and 100 mM KCl, the maximum binding reached 1 mol of HSP90 (dimer)/10 mol of actin, and the \(K_D\) was \(2.3 \times 10^{-6}\) M, assuming that the native \(M_r\) of HSP90 is 180,000 (our previous physicochemical study has shown that HSP90 exists in homodimeric form (19)). In 0.33 mM Mg\(^{2+}\), the maximum binding was the same, and the \(K_D\) increased to \(4.2 \times 10^{-6}\) M.

Fig. 3 shows the effect of tropomyosin on the binding of HSP90 to F-actin. With increasing concentrations of tropomyosin, the amount of HSP90 bound to F-actin decreased, and F-actin bound tropomyosin in place of HSP90. When increasing concentrations of HSP90 were added to actin solution in the presence of the fixed concentration of tropomyosin, the amount of tropomyosin bound to F-actin also decreased only slightly (data not shown). These results indicate that HSP90 and tropomyosin compete with each other for binding to F-actin and that F-actin binds tropomyosin more tightly than HSP90.

In the course of elucidating a control mechanism for the interaction of HSP90 with actin, we have found that Ca\(^{2+}\)-calmodulin inhibits the binding of HSP90 to F-actin. Fig. 4A shows that the inhibition by calmodulin in the presence of Ca\(^{2+}\) was dependent on the calmodulin concentration. About a 5-fold molar excess of calmodulin over the 90-kDa HSP90 polypeptide was required for the half-maximal inhibition. Fig. 4B shows the Ca\(^{2+}\) sensitivity of the binding of HSP90 to F-actin in the presence or absence of calmodulin. The binding was independent of Ca\(^{2+}\) concentration in the absence of calmodulin, whereas in the presence of calmodulin it was regulated by changes in Ca\(^{2+}\) concentration around \(10^{-6}\) to \(10^{-5}\) M. It was also observed here that calmodulin inhibits the binding to some extent even in the absence of Ca\(^{2+}\).

Whether calmodulin is capable of binding to HSP90 was examined by the equilibrium gel filtration method (27). A mixture of calmodulin and HSP90 was passed through Sephadex G-100 in a buffer solution containing the same concentration of calmodulin as described under "Materials and Methods." As shown in Fig. 5, a trough and peak in the profile of the calmodulin concentration appeared only in the presence of Ca\(^{2+}\). This clearly demonstrates that calmodulin binds to HSP90 in a Ca\(^{2+}\)-dependent fashion. The number of moles of calmodulin bound to HSP90 was determined from the area of

![Figure 1](image1.png)

**Fig. 1.** Binding of HSP90 to F-actin. Actin with or without HSP90 was incubated in the standard buffer solution and centrifuged. The pellet (A-C) and supernatant (a-c) fractions were analyzed by SDS-polyacrylamide gel electrophoresis. A, actin (0.49 mg/ml); B, actin + HSP90 (0.26 mg/ml); C, actin + HSP90 (0.52 mg/ml). HSP90 alone did not sediment under the conditions used.

![Figure 2](image2.png)

**Fig. 2.** Binding of increasing concentrations of HSP90 to F-actin. Actin (0.49 mg/ml) was incubated with increasing concentrations of HSP90 at 25 °C in 0.33 mM Mg\(^{2+}\) (C) or in 2 mM Mg\(^{2+}\), 100 mM KCl (○) and then processed for measurement of cosedimented HSP90 as described under "Materials and Methods." A, saturation curve. Binding assay was carried out in a buffer solution of 0.33 mM MgCl\(_2\), 0.7 mM EGTA, 0.04 mM ATP, and 30 mM PIPES, pH 6.7. B, Double-reciprocal plot ([actin]/[HSP90]) bound versus 1/[HSP90] free. ○, from the data shown in A; ●, from the data in the standard buffer solution (2 mM MgCl\(_2\) + 100 mM KCl).

![Figure 3](image3.png)

**Fig. 3.** Effect of tropomyosin on the binding of HSP90 to F-actin. Increasing concentrations of tropomyosin (TM) were mixed with actin solution (0.31 mg/ml) in the presence of a fixed concentration of HSP90 (0.39 mg/ml) in the standard buffer solution. The samples were centrifuged to sediment F-actin and then processed for measurement of cosedimented HSP90 and tropomyosin. ○, tropomyosin; ●, HSP90. The amount of bound HSP90 was expressed as the percent of the control value.
the binding of HSP90 to F-actin. Therefore, it may be concluded that Ca\(^{2+}\)-calmodulin inactivates the ability of HSP90 to bind to F-actin by forming a Ca\(^{2+}\)-calmodulin-HSP90 complex. The estimated \( K_D \) values for the binding of HSP90 to F-actin and calmodulin are relatively high, indicating that these interactions are not strong. However, it is quite likely that these interactions occur in cells because of abundance of these three proteins, actin, calmodulin, and HSP90. Yahara et al. (28) recently isolated a heat shock-resistant variant of Chinese hamster ovary cell line which specifically expresses a severalfold amount of HSP90 and shows altered properties as to morphology and cell adhesion. It is possible that the altered properties of this variant which might be due to some changes in cytoskeletal architecture would result from a high level of interaction between HSP90 and actin. The properties of HSP90 revealed here are apparently very similar to those of caldesmon, an abundant F-actin-binding protein of chicken gizzard smooth muscle whose F-actin binding activity can also be regulated in vitro by calmodulin (22, 29). However, their distribution in cultured cells is different. Thus, the immunoreactive forms of caldesmon which show considerable heterogeneity in their apparent molecular masses (120-140 or 71-77 kDa) are localized along the stress fibers and in the ruffling membranes (30, 31). On the other hand, HSP90 is localized in the ruffling membranes, but not in the stress fibers (19). In accord with their distribution, caldesmon does not compete with tropomyosin for binding to F-actin in vitro (30), whereas HSP90 bound to F-actin is easily replaced by tropomyosin (Fig. 3). Furthermore, physical-chemical properties of HSP90 are quite different from those of caldesmon (19, 22).

We have recently succeeded in isolating a relatively large amount of HSP90 from several mammalian tissues. The immunological cross-reactivity, peptide map, and polypeptide molecular weight of these HSP90s were found to be essentially the same as those of lymphoma HSP90 used here, and these HSP90s are also capable of binding to F-actin in vitro. Therefore, the properties of HSP90 revealed here might not be specific to the HSP90 of lymphoma cells, but common to every HSP90.

The Ca\(^{2+}\)-calmodulin-regulated binding of HSP90 to F-actin revealed here would be important, given that HSP90 transports biologically significant proteins such as p60\(^{ss}\) and steroid hormone receptor through interaction with actin as described in the Introduction. Furthermore, it is also possible that the Ca\(^{2+}\)-dependent binding of calmodulin to HSP90 regulates other functions of HSP90 which have not yet been identified.

REFERENCES


\(^{1}\) N. Yonezawa, E. Nishida, S. Koyasu, I. Yahara, and H. Sakai, manuscript in preparation.
Calmodulin and the 90-kDa Heat Shock Protein