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Two mammalian heat shock proteins, HSP90 and HSP100, are actin-binding proteins

(stress proteins/membrane ruffles/protein transportation)

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ABSTRACT **Two high molecular weight heat shock proteins, HSP90 (M_r, 90,000) and HSP100 (M_r, 100,000), were separately purified from extracts of cultured cells of a mouse lymphoma cell line, L5178Y. Both of the HSPs exist in homodimeric form under physiological conditions. Their physicochemical properties are quite similar to each other. Each of the purified HSPs was shown to coprecipitate with rabbit skeletal muscle actin under actin-polymerizing conditions. Both HSP90 and HSP100 increased the low-shear viscosity of filamentous actin solutions in a dose-dependent manner, which suggests that these HSPs cross-link actin filaments. Although some molecular properties and the effects described above on actin solution of HSP90 and HSP100 resemble those of α-actinin, the HSPs were distinguished from α-actinin by various means, including visualization of molecular shapes by electron microscopy with the aid of the low-angle rotary shadowing technique. Immunofluorescence staining by specific antisera against HSP90 revealed that HSP90 was localized in ruffling membranes in addition to the cytoplasmic space.**

Both eukaryotic and prokaryotic cells synthesize a class of specific proteins called heat shock proteins (HSPs) in response to various environmental insults or harmful drugs, such as increased temperature, metabolic inhibitors, amino acid analogues, or transition series metals (1, 2). Species of HSPs and their amino acid sequences have been conserved during evolution, which suggests that functions of HSPs are essential for living organisms. Because of the specific localization of some HSPs, it is supposed that these HSPs might function in the nucleus in protection of chromatin and related structures against heat shock and other stresses (3–5). Other results suggest that a certain HSP is present in association with cytoskeletal frameworks (6). Apart from the specific localizations of HSPs, several HSPs have recently been identified as previously known proteins, although relationship of their functions to heat shock responses has not been clarified yet (7–9). Among HSPs of higher vertebrates, HSP90 has been shown to have intriguing properties: (*i*) HSP90 forms a tripartite complex together with $pp60^{src}$, the product of v-*src*, and a phosphoprotein of M_r 50,000 in cultured avian cells (10–12). Other viral transforming proteins bearing tyrosine-specific protein kinase activity, $pp140^{fps}$ and $pp94^{yes}$, are also associated transiently with HSP90 (13). (*ii*) More recently, it was shown that HSP90 is a steroid hormone receptor-binding protein (14–16). Both of the observations suggest that HSP90 is a carrier protein for molecules bearing biological key functions. We report here that HSP90 and HSP100 are actin-binding proteins. This finding would be important if HSP90 and HSP100 are really involved in the intracellular transport system of various functional proteins.

MATERIALS AND METHODS

Materials. Porcine insulin was obtained from Eli Lilly; L-[^{35}S]methionine (1000–1300 Ci/mmol; 1 Ci = 37 GBq) was from Amersham; Bio-Gel HTP was from Bio-Rad; DEAE-cellulose DE52 was from Whatman; Sephadex G-100, Sephacryl S300, and Mono Q were from Pharmacia. RPMI 1640 medium and modified Eagle's medium were generally supplemented with 10 mM Hepes/0.2% sodium bicarbonate/penicillin G (100 units/ml)/kanamycin (0.2 mg/ml).

Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (17) and was further purified by gel filtration on Sephadex G-100 as described (18). Smooth muscle α-actinin was purified from bovine stomach as described (19). Purified rat actinogelin (20, 21) and rhodamine isothiocyanate-conjugated phalloin (22) were gifts from A. Asano (Sapporo Medical College, Sapporo, Japan) and from Th. Wieland (Max Planck Institut, Heidelberg, F.R.G.), respectively.

Cells and Growth Conditions. L5178Y, a mouse lymphoma cell line, was used as the source of HSP90 and HSP100 in this study. L5178Y cells were cultured in RPMI 1640 medium containing 10% fetal calf serum. Human epidermoid carcinoma cell line KB was provided by K. Goshima (Nagoya University, Nagoya, Japan) and cultured in modified Eagle's medium containing 10% calf serum. Ruffling membranes were induced in KB cells by insulin according to the method of Goshima *et al.* (23) with slight modifications (43).

Analysis of Proteins. $NaDodSO_4$/PAGE and two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPHGE)/$NaDodSO_4$/PAGE were carried out as described elsewhere (24, 25). Peptide mapping with *Staphylococcus aureus* V8 protease was performed according to the method of Cleveland *et al.* (26).

Immunofluorescence Staining. Preparation of rabbit antisera against HSP90 and examinations of their specificity will be described elsewhere (unpublished data). Immunofluorescence staining was performed as described (27, 28). Fixed and permeabilized cells were incubated with diluted (1:500) anti-HSP90 antiserum and subsequently with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, after which the cells were further incubated with rhodamine isothiocyanate-conjugated phalloin to visualize actin filaments.

Interaction of HSPs with Actin Filaments. For coprecipitation experiments, actin at the indicated concentrations was incubated at 25°C for 1 hr in a buffer solution consisting of 2

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Abbreviations: HSP, heat shock protein; NEPHGE, nonequilibrium pH gradient gel electrophoresis.

mM $MgCl_2$/100 mM KCl/0.6 mM EGTA/0.04 mM ATP/30 mM Pipes, pH 6.7, in the presence or absence of HSP90 or HSP100. The mixtures were then centrifuged at 100,000 × *g* for 2 hr. Proteins contained in the supernatants and those in the pellets were analyzed by $NaDodSO_4$/PAGE.

Low-shear viscosity was measured with a falling ball device (29). Various concentrations of HSP90 and HSP100 were mixed with monomeric actin in the polymerization buffer described above, after which the mixtures were drawn up into capillary tubes. Polymerization of actin was allowed at 25°C for 30 min. After the capillaries were set up at an angle of 55°, apparent low-shear viscosity was determined by measuring time required for a stainless steel ball to fall through a constant path along the capillary.

Low-Angle Rotary Shadowing. Samples were dialyzed against 0.1 M ammonium acetate (pH 7.0) and prepared at a protein concentration of 30 μg/ml in the same salt solution containing 60% (vol/vol) glycerol. Each sample was sprayed onto a freshly cleaved mica surface, after which the surface was shadowed with platinum/carbon on a rotary stage of freeze fracture and etching device (FD-3; Eiko Engineering, Mito, Japan) at an angle of 4°, and then coated with carbon. The specimens were examined in a JEOL JEM 100-C electron microscope at 80 kV.

RESULTS

Purification of HSP90 and HSP100. Expression of HSPs at the basal level in cells growing under normal conditions differs between the homeotherm and the heterotherm. Differently from yeast and *Drosophila* cells, growing avian and mammalian cells synthesize some HSPs without exposure to heat shock or other stresses, although the extent of expression of these HSPs was further increased upon exposure to stress (31). We have found that cultured cells of mouse lymphoma cell line L5178Y express HSP90 to a large extent (1–2% of the total proteins). Thus, two heat shock proteins, HSP90 and HSP100, were purified from cultured L5178Y cells. Three initial steps for purification of the two HSPs with columns of DEAE-cellulose, hydroxylapatite, and Sephacryl S300 generally followed the methods described by Welch and Feramisco (32). As was previously shown, HSP90 and HSP100 were copurified through these purification steps (32). Fractions containing HSP90 and HSP100 were dialyzed against 50 mM Tris·HCl (pH 7.6) containing 10 mM NaCl, and applied a Pharmacia automated FPLC system equipped with a Mono Q column that had been equilibrated with the same buffer. HSP90 and HSP100 were separately eluted at NaCl concentrations of 0.5 and 0.6 M, respectively (Fig. 1*A*). Each of the purified proteins was coelectrophoresed in two-dimensional gels with the total proteins of L5178Y cells to examine their identity (Fig. 2). The results clearly showed that the two purified proteins were superimposed over two heat shock-inducible proteins, HSP90 and HSP100, respectively. Peptide maps of the two HSPs by partial digestion with staphylococcal V8 protease differed from each other (Fig. 1*B*). Physicochemical studies indicated that both of the two HSPs are dimeric and possess large frictional ratios (f/f_0) of ≈1.8 (Table 1).

Immunofluorescence Staining of HSP90. The distribution of HSP90 in cultured cells with a flat morphology was examined by the indirect immunofluorescence method (27, 28). As shown with KB cells, in which membrane ruffles were induced in response to insulin (23), a bulk of HSP90 was present in the cytoplasm around the nucleus and, in addition, a part of HSP90 was localized in ruffling membranes (Fig. 3). Remarkably high intensity of the fluorescence labeling in membrane ruffles strongly suggests an abundance of HSP90 in these regions. Double labeling of the same cells with rhodamine isothiocyanate-conjugated phalloin clearly demonstrated that quantities of filamentous actin molecules were present in ruffles too (Fig. 3). Localization of HSP90 in membrane ruffles was observed also with other cell types, including mouse C3H-2K fibroblasts and rat 3Y1 fibroblast cells (data not shown). However, HSP90 did not appear to be associated with stress fibers, which are composed of bundles of actin filaments. These observations led us to assume that HSP90 might interact with networks of actin filaments.

Interactions of HSP90 and HSP100 with Actin. We have examined the above possibility *in vitro*. Rabbit skeletal muscle actin was incubated under the condition of actin polymerization in the presence or absence of HSP90. Polymerized actin was then pelleted by centrifugation. We found that a part of HSP90 coprecipitated with polymerized actin (Fig. 4*A*), whereas an incubation of HSP90 alone under the same condition did not cause significant precipitation of the protein (data not shown). When another soluble protein, bovine serum albumin at 1 mg/ml, was incubated with actin under the same condition, no coprecipitation of albumin with polymerized actin was detected, indicating that the coprecipitation of HSPs and actin filaments was not due to nonspecific trapping. When HSP100 was added to the actin solution instead of HSP90, we found that HSP100 coprecipitated with polymerized actin too. HSP100 alone did not cause precipitation under the same condition (data not shown).

To examine the mode of interaction between actin filaments and these two HSPs, we determined the effects of HSPs on low-shear viscosity of actin filaments. As shown in Fig. 4*B*, both of the HSPs increased the low shear viscosity of actin filaments in a dose-dependent manner. Interaction between these HSPs and actin filaments was not affected by Ca^{2+} (Fig. 4*B*). These results indicate that both HSP90 and

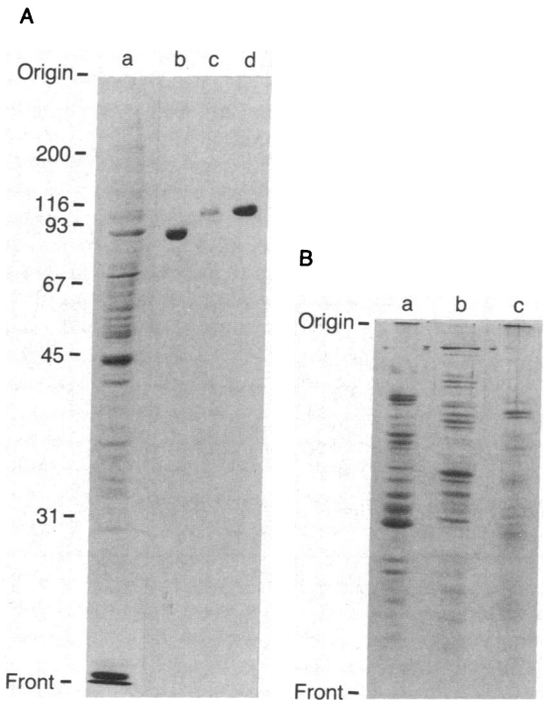

FIG. 1. Purification of HSP90 and HSP100. (*A*) $NaDodSO_4$/PAGE of purified HSP90, HSP100, and the nonmuscle α-actinin, actinogelin. Lanes: a, L5178Y extract; b, HSP90; c, HSP100; d, actinogelin electrophoresed in $NaDodSO_4$/PAGE and stained with Coomassie brilliant blue. Numbers represent $M_r \times 10^{-3}$. (*B*) Peptide mapping analysis. Five micrograms of HSP90 (lane a), actinogelin (lane b), and HSP100 (lane c) was partially digested with 5 ng of staphylococcal V8 protease as described (26). After $NaDodSO_4$/PAGE, the gel was stained with Coomassie brilliant blue.

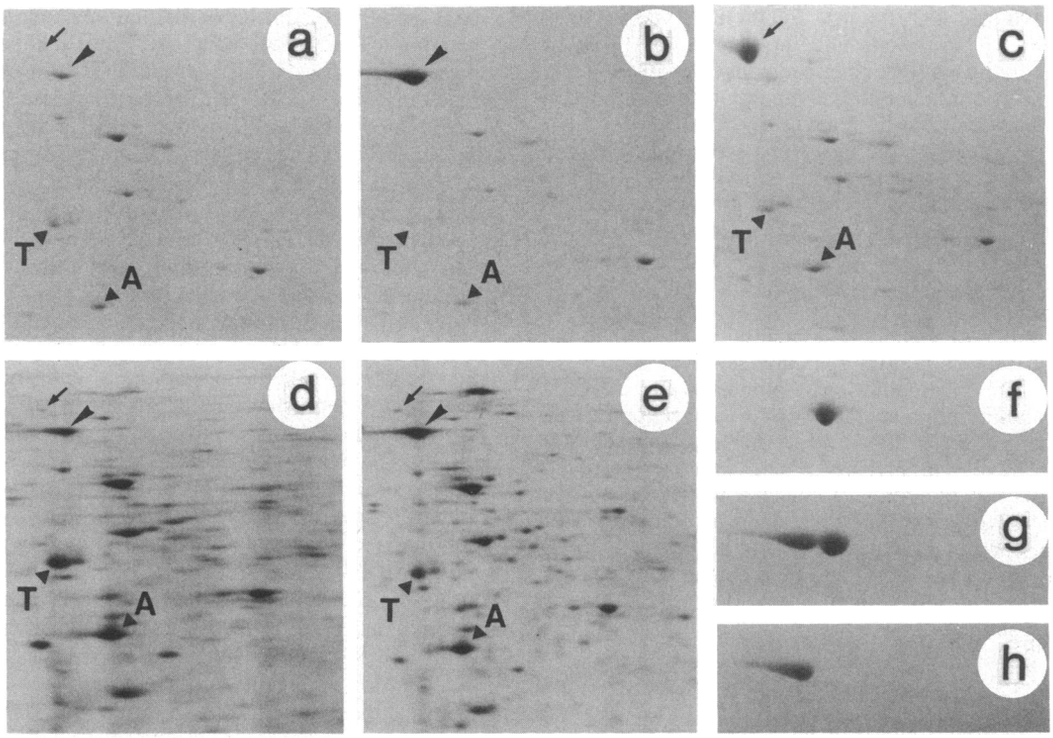

FIG. 2. Identification of the purified proteins as HSP90 and HSP100. (*a*) L5178Y total cell extract; (*b*) a mixture of purified HSP90 and L5178Y total cell extract; and (*c*) a mixture of purified HSP100 and L5178Y total cell extract, were analyzed by two-dimensional NEPHGE/NaDodSO$_4$/PAGE. L5178Y cells were labeled with L-[^{35}S]methionine for 1 hr at 37°C (*d*) or 42°C (*e*), and total cell extracts were applied to two-dimensional NEPHGE/NaDodSO$_4$/PAGE. After electrophoresis, the gels were exposed to prefogged Kodak X-Omat AR x-ray films. (*f*) HSP100; (*g*) a mixture of HSP100 and actinogelin; and (*h*) actinogelin, were applied to two-dimensional NEPHGE/NaDodSO$_4$/PAGE and were stained with Coomassie brilliant blue. Arrows and arrowheads indicate HSP100 and HSP90, respectively. A and T indicate actin and tubulin, respectively.

HSP100 are included in a category of actin-binding proteins that have the ability to cross-link actin filaments.

Molecular Shape Observed by Electron Microscopy. HSP90 and HSP100 possess large frictional ratios (f/f_0), indicating that both HSPs are nonspherical proteins. Thus, molecular shapes of HSP90 and HSP100 were examined by electron microscopy with the aid of the low-angle rotary shadowing technique (30). As shown in Fig. 5 (*a* and *b*) HSP90 and HSP100 were indistinguishable in their molecular shape and appeared to be composed of two spherical domains connected with each other by a flexible junction. α-Actinin is a typical actin-binding protein, which can cross-link actin filaments. We have, therefore, compared the two HSPs and the α-actinin isoproteins in their molecular shape (Fig. 5*c* and *d*). Differently from HSP90 and HSP100, actinogelin, a nonmuscle α-actinin, and smooth muscle α-actinin appeared to be rigid rods consisting of two identical subunits associated in a side-by-side fashion as reported (21).

Table 1. Physicochemical properties of HSP90, HSP100, and α-actinin

	HSP90	HSP100	α-Actinin*
Sedimentation coefficient ($s^0_{20,w}$)	6.2S	6.5S	6.8S
Stokes radius, nm	6.5	6.8	7.3
Native M_r†	170,000	185,000	209,000
M_r from NaDodSO$_4$/PAGE	90,000	100,000	103,000
Form	Dimer	Dimer	Dimer
Frictional ratio (f/f_0)‡	1.8	1.8	1.9

*Data for α-actinin of rabbit macrophage (33).
†Calculated from the values of sedimentation coefficient and Stokes radius. Partial specific volume ($\bar{v}_0$) was assumed to be 0.73.
‡f/f_0 values here included the contribution of solvation.

DISCUSSION

We have described purification of HSP90 and HSP100 and showed that both HSPs are actin-binding proteins that can cross-link actin filaments. Both HSP90 and HSP100 resemble α-actinin; they have similar physicochemical parameters (Table 1) and the ability to cross-link actin filaments. However, the HSPs obviously differ from actinogelin or other nonmuscle α-actinin as the two high molecular weight HSPs bind actin filaments independently of the presence of Ca^{2+}, whereas nonmuscle α-actinin interacts with actin filaments in a Ca^{2+}-dependent manner (20, 33, 34). Rabbit antiserum against chicken gizzard α-actinin did not cross-react with these HSPs, and rabbit antiserum against HSP90 did not cross-react with α-actinin (unpublished observations). In addition, the two HSPs differ from α-actinin in their molecular shape as revealed by electron microscopy. These results indicated that these two HSPs are different actin-binding proteins.

The binding of HSP90 to actin filaments was saturable, which suggests that this binding was specific. The K_d value calculated from the binding experiments indicated that an interaction between HSP90 and actin is relatively weak ($K_d = 2.3 \times 10^{-6}$ M) as compared to an interaction between α-actinin and actin ($K_d = 2 \times 10^{-7}$ M; ref. 33). We have recently found that HSP90 attached to actin filaments was readily replaced by tropomyosin (44), which would explain the fact that HSP90 is not associated with stress fibers in fibroblastic cells (see above). Despite the low affinity of HSP90 to actin, these two proteins may possibly interact with each other in cultured mammalian cells because of an

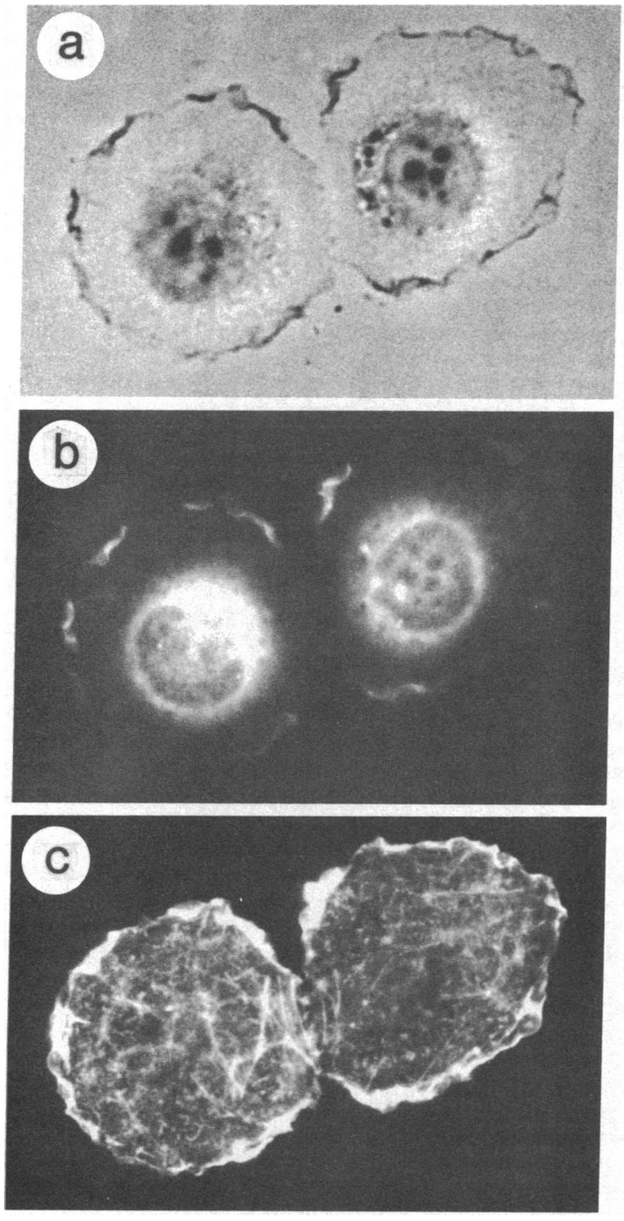

FIG. 3. Visualization of HSP90 and actin filaments in insulin-stimulated KB cells. HSP90 and actin filaments were visualized by the immunofluorescence double-staining method. (*a*) Phase contrast. (*b*) HSP90 labeled by rabbit anti-HSP90 antiserum followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody. (*c*) Actin filaments labeled by rhodamine isothiocyanate-conjugated phalloin.

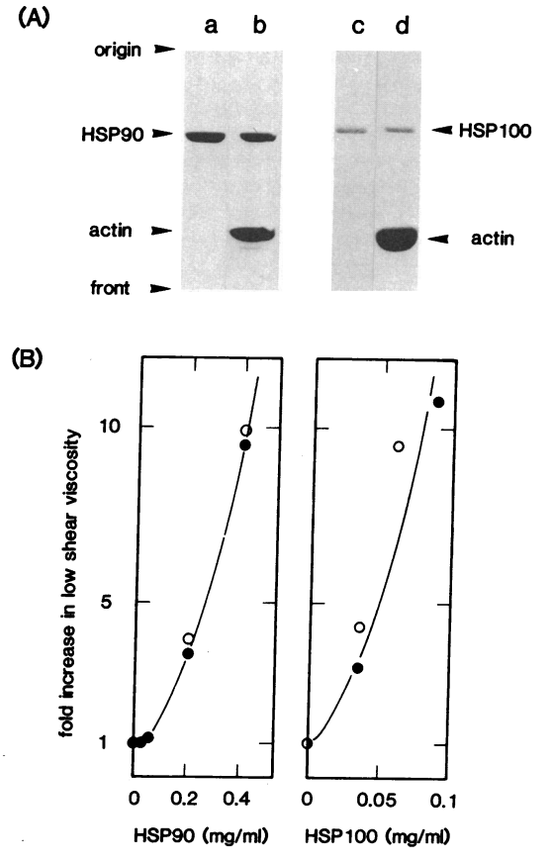

FIG. 4. Interaction of HSPs and actin. (*A*) Coprecipitation of HSPs with actin filaments. After polymerization of actin with HSP90 (lanes a and b) or HSP100 (lanes c and d), samples were centrifuged and the supernatant (lanes a and c) and pellet (lanes b and d) were analyzed by $NaDodSO_4$/PAGE. Lanes: a and b, actin (0.31 mg/ml) and HSP90 (0.39 mg/ml); c and d, actin (0.51 mg/ml) and HSP100 (0.17 mg/ml). (*B*) Effect of HSPs on low-shear viscosity of actin filaments. Increasing concentration of HSP90 (*Left*) or HSP100 (*Right*) were mixed with actin (final concentration, 0.24 mg/ml) either with (○) or without (●) Ca^{2+} (●, 0.6 mM EGTA; ○, 0.6 mM EGTA/0.8 mM $CaCl_2$), and the apparent low-shear viscosity was determined. The data are normalized to the viscosity of actin alone.

abundance of these proteins. The concentration of HSP90 in L5178Y cells was estimated to be ≈1 mg/ml (≈10^{-5} M) from the two-dimensional gel electrophoretic pattern (Fig. 2) and is enough for interaction with actin. As shown in Fig. 3, both actin and HSP90 are preferentially localized in ruffling membranes of substrate-adhered cells, where they might interact with each other.

We have recently isolated a heat shock-resistant variant of a Chinese hamster ovary (CHO) cell line that specifically expresses severalfold amounts of HSP90 and has a relatively flat morphology as compared to the parental strain (35). In addition, the variant always forms larger colonies than those of the parental CHO strain, although these two strains proliferate at the same growth rate. This fact is attributable to a relatively high motility associated with the variant as compared to the parental strain. Since HSP90 is the only protein that is significantly overexpressed in the variant, the different properties revealed by the CHO variant in cell morphology and motility would be a consequence of a relatively high level of interaction between actin and HSP90 in the variant. More recently, we have found that calmodulin modulated the interaction of HSP90 with actin *in vitro* in a Ca^{2+}-dependent manner (44), suggesting that Ca^{2+} may be a factor that regulates the interaction between HSP90 and actin *in vivo*.

As shown above, HSP90 and HSP100 are quite similar in their molecular profiles, including physicochemical parameters, molecular shapes revealed by electron microscopy, and the ability to bind actin filaments. However, these two HSPs have been distinguished by two-dimensional NEPHGE/$NaDodSO_4$/PAGE and peptide mapping. Furthermore, different properties of the two proteins have previously been documented. First, HSP100 is highly glycosylated, whereas HSP90 is not (36). Second, antisera raised against HSP90 did not cross-react to HSP100 (data not shown, ref. 37) and vice versa (36). Furthermore, unlike HSP90, HSP100 has been shown to be localized in Golgi apparatus and to translocate into the nucleus upon heat shock (36). In this regard, of interest are the recent findings that cytoplasmic actin was induced to translocate into nucleus and to form paracrystal-like structures when cultured cells were exposed to increased

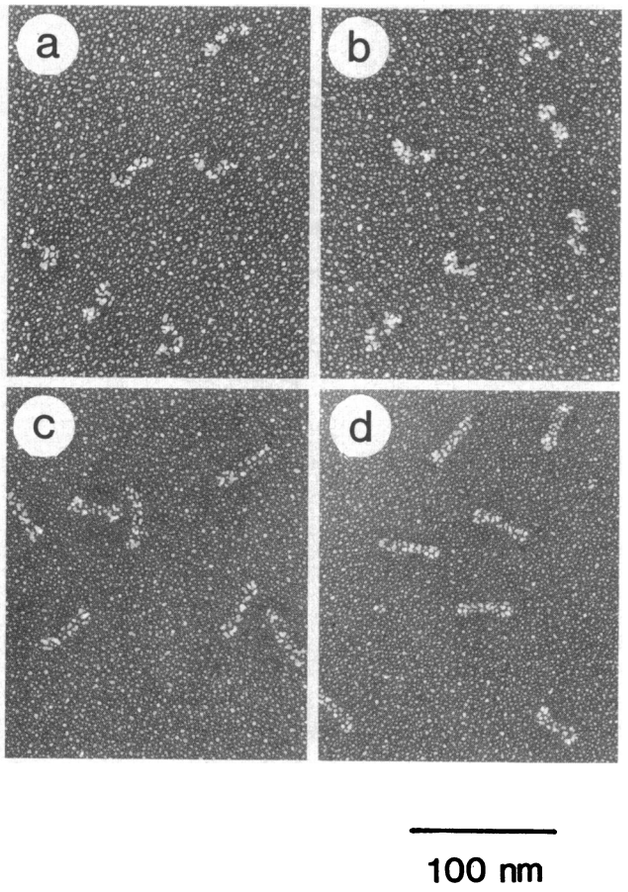

FIG. 5. Molecular shape of HSPs and α-actinins examined by low-angle rotary shadowing. (*a*) HSP90; (*b*) HSP100; (*c*) actinogelin; (*d*) smooth muscle α-actinin were observed by low-angle rotary shadowing of platinum/carbon as described (30).

temperatures (38, 39). We have no evidence for interaction between HSP100 and actin in nuclei of heat shocked cells, however.

It has been suggested that HSP90 forms intracellular complexes with functionally key molecules including $pp60^{src}$ and steroid hormone receptors (10–16). The association between HSP90 and $pp60^{src}$ ($pp140^{fps}$ or $pp94^{yes}$) together with cellular phosphoprotein pp50 is transient and is thought to be involved in transportation of $pp60^{src}$ from the cytoplasm to the plasma membrane where the src protein expresses its function (40, 41). Various steroid hormone receptors are present in the cytoplasm and translocate into nuclei after activation by respective steroid hormones (42). On the basis of biochemical and immunological identification of protein species, it has recently been shown that untransformed or inactive steroid hormone receptor complexes contain HSP90 as their component and that activation of steroid hormone receptor complexes accompanies dissociation of HSP90 from the complexes (14–16). In this case too, HSP90 may function in transportation of hormone receptor complexes to the nucleus. If HSP90 is a common carrier protein for transportation of biologically key functional molecules such as $pp60^{src}$ or steroid hormone, an interaction between HSP90 and actin shown in the present study would have biological significance; for instance, a nonmuscle actomyosin system may provide mechanical forces for the transportation via interacting with the HSP90 moiety of the complexes.

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