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INTERACTION OF HEAT SHOCK PROTEIN (hsp90) WITH THE CYTOSKELETON: POTENTIAL IMPLICATIONS IN INTRACELLULAR TRANSPORT

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SUMMARY

• 7/i *this article we will summarize the details concerning the association of'90kDheai shock prorein (hsp90) with cytoskeletal structures and we will discuss the potential involvement t>fihese interactions in the translocation of steroid hormone receptors to ihece.ll nucleus.*

In cultured mammalian cells hsp90 has been found to be colocalized with both microtubules and cytokeratin intermediate filaments, whereas no association with actin filaments andvimentinintermedhite filaments has been established.

Tlie colocaHytiioH of hsp90 with microtubiiles and cytokeratin^ in intact cells ri:-es the possibility thai cwoxkeletal structures couldJH.'ii'.ed as "rails"for the direct movement of the steroid hormone receptor via association-dissociation with hsp90 molecules jroui the cy ioplasmic site. ofs\ntliasis to the nuclear site, of action.

INTRODUCTION

• The cytoskeleion, the integral structure network of the cell, has been previously compared loihc bonus and muscles of" [he body. However, its function is much more complex in many \vays al I owing participation in the regulation of cell shape, cell movement, cell division, endocytosis and adhesion. The cyLoskeleton is composed of three well characteri/ed classes of filamentous structures: (i) microtubulcs which are hollow cylinders of approximately 25nm outer diameter and indefinite length; they are constructed from identical subunils of tubulin, a globular protein of approxiinately 1 H)kD.(ii) actin filaments which are 6-8 nm in diameter; they have the basic structure of double-stranded helix and are made from of assembled uctin molecules and the associated tropomyosin and troponin. and (iii) intermediate filaments which are composed of different types of proteins assembled in structures of 8-1 I nm in diameter; there are live major types orinlermedtate filaments described according to their proiei n subunits: vimentin, desmin. cytokeratins. neurofi!amen!pro;eins (Nt'-I ..NF--VLNF-H), andglial fibrallary acidic protein (GFAP), which arc specifically expressed in particular cell types.

The research on the cellular organization and distribution of the filamentous STRUCTURES was made possible through the application of labelled antibody procedures. The first of these studies some 20 years ago used antibodies against actin to visualise actin filamems (1) similar studies with ami bodies against tubulin ( ) and intermediate fiiamenl proteins (5,6) follwed soon. Through (he foiJ;nvin2 years the irnm ofluoreseence mi-

croscopy techniques were fully established providing a convenient and useful tool for determining the organization and distribution of cytoskeletal proteins and structures, as well as their association with each other or with other cellular components.

The analysis of cytoskeletal preparations supports the understanding that the three major cytoskeletal structures are intercomiecied and their functions are coordinated.

Compared to the remarkable progress at the structural level, our knowledge of the biochemical basis of these interactions as well as of the physiological role of the cytoskeletal elements are very poor. One of the most striking features of the concept of interaction of cytoskeletal structures, is their involvement in intracellular transport processes. It is a challenging aim to searchfor lines of evidence regarding theuse of cytoskeletal structures in the intracciluiar protein transpor.. Studies of thecolocaluationof hsp90wiih the cytoskeletal proteins provide aninteresting area to approach such an aim.

The cellular function of hsp90 is still unknown (7), despite of its ubiquitous presence among eukaryotic cells. Hsp90 associates with the oncoprotein $pp60^{SIL}$ as well as with some other sarcoma virus transforming proteins (8-10). Hsp90 is also associated with steroid receptors in their unuransformed, non-DN A-binding state in cytosol preparation (11-13). Two functions have been described to receptor-bound hsp90: (i) masking the receptor DNA binding domain, and (ii) maintenance of the ligand binding domain in a functional hormone-bind ing conformation (14). Thus, modulation of important functions of the steroid receptors has been attributed to hsp90. including prevention of DNA binding and optimization of transcriptional activity.

Searching to attribute a biological role and function for hsp90, efforts were undertaken recently to study the interaction of this protein with the cytoskeleton. Isolated hsp90 showed the ability to bind to F-actin (15.16). while no association of the protein with actin filaments could be demonstrated in intact cells. Using immunoflu ore seen ce microscopy. =he colocalization of hsp90 with microtubules has been shown in cytosolic preparations of several mammalian cell lines (17.18). Moreover, the interaction of hsp90 with cytokcratin intermediate filaments has been also postulated in a human endometrial adenocarcinoma cell line (19).

In the present paper we will summarize the details of our studies concerning the association of hsp90 with cytoskeletal proteins and structures and we will discuss the potential involvement of this interaction in the intraccllular translocation of steroid hormone re ceptors.

hsp90 EXPRESSION IN VARIOUS CELL TYPES

• A wide range of cells, when exposed to elevated temperatures or otherenvironmental stresses, synthesize specific proteins called heal shock proteins (20.21). They play important roles in cellular functions, suggesting that they are es^entialforiiving cells (22-24). Using immunofluorescence microscopy techniques. hsp90 has been found almost exclusively in the cytoplasm of chicken embryo fibroblasts (25). HeLa cells (.26), and Drosophila cells (27). Recent studies demonstrated the presence of hsp90 also in the nucleus (25.28,29).

The determination of the cellular localization of hsp90 and particularly of its colocalization with filamentous structures could provide us with importantclues for its function. Collier and Schlesinger (25) reported afibrous distribution of hsp90in less dense edges of chicken fibroblasts. Sanchezetal(17} showed that monoclonal antibodies against tubulin and hsp90 could be used to immunoadsorb hsp90 and tubulin. from the cytoplasm of L929 fibroblasts. Moreover. they reported a colocalizution of hsp90 with cytoplasmic microtubules.

However, other groups using two distinct monoclonal antibodies, which immunoprecipitate both free hsp90 and hsp90 complexed with other proteins, reported no immune-specific coadsorption of either a- or [i-tubuiin (30).

Conclusive data concerning an association of hsp90 with filamentous structures were provided only recently (18.19). If hsp90 is colocali zed with cytoskeletal

elements in their filamentous form, it should also be presentin the Triton-insoluble cytoskeleton. Based on such an assumption, Fostinisctal (19) prepared this fraction from Ishikawa human endometriai cells. Indeed, when these preparations were stained with anti-hsp90 antibodies, filamentous structures were clearly-observed, indicating that hsp90 is present in the cytosol- and membrane-free cellular fragment of cytoskeleton (Fig. 1).

hsp90 DDES MOT SEEM TO ASSOCIATE WITH F-ACTIN

• Koyasu ct al first reported that hsp90 purified from a mouse lymphooma cell line showed the ability to bind to purified F-actin (15). This binding which was saturable in a molar[1] ratio of about 1 hsp90 dimmer to 10 actin molecules was proposed to be $Ca^{:+}$dependent, since it was regulated by calmoduHn (16). It was concluded that $Ca^{:+}$-calmodulin inactivates the ability of hsp90 to bind to F-actin by forming a Ca^{-+}-calmodulin-hsp90 complex. The K_D

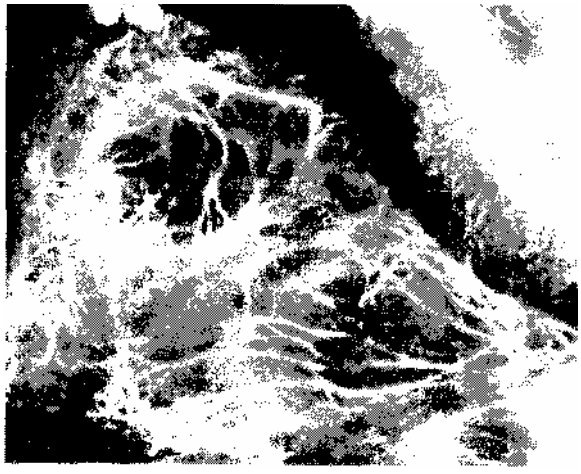

Figure 1. Imnwnofliiorescence microscopy in preparations of the Triton-insoluble cytoskeleton of Ishikawa cells: Immunostaining of hsp90 with anti-hsp90 antibiodies, clearly shoeing filamentous structures.

values for the binding of hsp90 to F-actin were found to be relatively high, indicating a week interaction (16). Moreover, hsp90 was dissociated from F-actin by the binding of tropomyosin to F-actin. However, this association was demons trated only with purified hsp90andF-ac tin, when both components were present in solution in high concentrations'. No localization of hsp90 with stress fibers could be demonstrated in cytoso] preparations or in intact cells (-15). Thus, the questionremained open if hsp90really associates with actin filaments in living cells.

Using double immunofluorescence staining, a much more detailed study on the cellular colocalization of hsp90 with actin filaments has been performed by Fostinis et al (19). In this work. Ishikawa human endometriai cells were treated with $1O^{5}M$ cytochalasin B in order to destroying actin filaments; this was followed by double staining withrhodamine phalloidin and anti-hsp90 antibodies. Under these experimental conditions the aclin filament staining of the cells totally disappeared, while residual filamentous structures were labelled with anti-hsp90 antibodies (19). From these results it was concluded that hsp90 is not associated with actin filaments in cultured Ishikawa cells. These observations supported the suggestion lhat the *in vitro* reported binding of hsp90 to F-actin does not reflect the real situation in the living cell. Thus, the hypothesis regarding the involvement of hsp90 in the transport of biologically important proteins, such as steroid hormone receptors, through their interaction with actin (16), seems to be unlikely,

THE COLOCALIZATIOH OF hsp90 WITH MICROTUBULES

• Recent studies on the coadsorpuon of hsp90 and tubulin in cy tosolic preparations with several monoclonal ami-hsp90 and anti-tubulin antibodies provide unconclusivedata (17,30). It was previously reported that hsp90 has a rather diffused cytoplasmic distribution without relation to filamentous structure^ (25,27.31). However, some of these studies were performed with round-shaped cells having *z* relatively small cytoplasmic volume, such as Drosophila or HeLa cells (27,31), where microtubules are much more difficult to be observed. Moreover, the mitotic spindle could not be been observed because these cells were not dividing.

distribution of hsp90 in imactcultured PiKj cells using indirect immunofluorescence microscopy with a monoclonal unti-hsp^{c}X)untibody(AC88). They showed that in intcrphasc cells the pattern of the fluorescence staining was disiributed in a filamentous way throughout the cytoplasm and in metaphase cells ii was located on the mitolic spindle. In addition these studies have further provided biochemical evidence for an association of hsp90 with tubulin. Indeed, anti-tubuiin and ACS8 antibodies were able to immunoadsorb hsp9() and tubulin, respectively, from ihecytosol of 1 .-cells. These results suggested that hsp90 seems to associate \\1lhlubulin-containing complexes in intact Ft K, cells. Recently, the same group presented a more detailed study on the colocali/aiion of hsp90 with niicrotnbules in mammalian cells (18). In this report, two antibodies against hsp90. a monoclonal (AC88) and a polyclonal, were used to study the localization of hsp90 in rat endotheiial and PtK, epithelial cells. B oth antibodies produced imimmofluorescence pattern: identical to microtubular networks, indicating tha hsp90 binds to microtubule in all stages of the eel cycle. In all cells studied, AC88 is found on ihi microtubules of interphase cells. Moreover, the mi toiic apparatus was also labelled by AC88 and b; polyclonal antibody in all stages of mitosis. Thf hsp90-microtubules association was further sup ported by the finding that labelling of these cyioplas mic structures was eliminated when ariti-hsp90 an libodies were preincubated with purified hsp9().

The colocalization of hsp90 with microtubules wa also recently shown in Ishikav, a cells (19,}. Using . polyclonal ami-hsp90 antibody, a filamentous mor phology of hsp90 staining and a coloration of th mitolic spindle was clearly observable in ihese cell (Fig. 2). thus confirming ihe previously publish^ data(17,18).

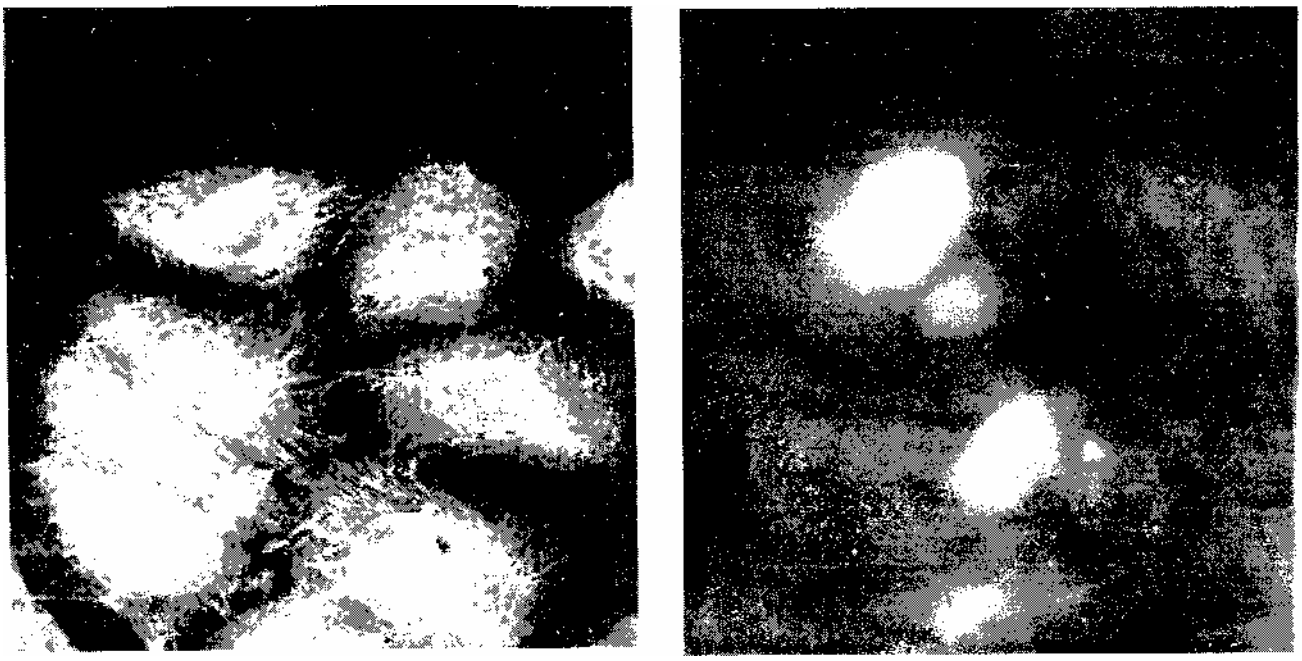

***Figure** 2. Immunofluorescence microscopy affixed Ishikawu cells stained with anti-hsp90 antibodies. Demonstration ofcytoplnunicfilamentous stmcntresUi). and- the mitolic spindle (b).*

IMMUNOSTAINING OF tispQO IN THE PRESENCE OF CYTOSKELETAl INHIBITORS

M Redmond *ei* al (18) reported that treatment of rat enctothelial cells with the antitubulin agent coleemid caused the disappearance of microtubules, while an identical weak labeling was also observed when cells were stained wilh anti-hsp90 antibodies. suggesting that most probably hsp90 coiocali/.es only \vithmicrotubules. However, Fostinisei al (19) showed that a clear residual filamentous staining with anti hsp90 antibodies was still present, when the cells were incubated with colchicine or triethyllead. two different and well established antitubulin agents (33.34). Furthermore, under exactly the same experimental conditions a total breakdown of the mierotubules was demonstrated, when cells were stained for tubulin. From these results it was concluded that hsp90 most probably associates not only wilh mierotubules but also wilh structures resistant to antitubulin agents.

What could be the interpretation of these contradictory reports?

Redmond et al (18) used an overnight cell fixation procedure in formaldehyde, followed by the immunostaining of the proteins. This fixation procedure, however, seems to be very drastic, it has been shown to favour the rearrangement of some proteins or even the falling off in the staining ofcytoskeleial structures (32), Moreover, Fostinis etal (19) reported that using the formaldehyde method for longer then 10 minutes to fix Ishikawa cells, an increasing falling off in the immunostaining of mierotubules and vimentin intermediate filaments became evident. Thus, the residual hsp9Olabelled structures still present in Ishikawa cells treated by antitubufins are most probably visualized because of using quick and mild fixation procedures. In consequence, the use of a mild cell fixation technique seems to be very important to preserve the labelled filaments.

STUDYING THE INTERACTION OF hsp90 WITH INTERMEDIATE FILAMENT PROTEINS

• It seems that hsp90 associates not only with mierotubules but most probably also with other filamentous structures. Since all reported data point out that hsp90 does not colocalize with actin filaments, ihe interaction of this protein with intermediate filament structures should be possible. Owing 10 the failure of specific inhibitors for intermediate filament proteins, this hypothesis is difficult to be tested. Actin filament and microtubule inhibitors, inducing structural rearrangements in the organization of this cytoskeletal protein family, can be used (35,36).

This was recently attempted by Fostinis etal (19), using triethyllead, colchicine or cytochalasin B. and mixtures of these inhibitors to study the interaction of hsp90 with vimentin and cytokeratin intermediate filaments. The concentration of the cytoskeletal inhibitors used were in all cases, higher of those causing the full breakdown of mierotubules and actin filaments. This was essential to exclude any remaining hsp90 labelling with mierotubules still intact.

hspSO SEEMS TO NOT ASSOCIATE WITH VIMENTIN INTERMEDIATE FILAMENTS

• When cells treated by triethyllead were stained for vimentin. a clear rearrangement of the filaments became evident, presenting all morphological characteristics described for this interaction (35). However, under the same experimental conditions the immunostaining of hsp90 was noticeably different. These data indicate that hsp90 does not associate with vimentin intermediate filaments. Double staining immunofluorescence experiments exposing both staining patterns on the same film plaque further supported this hypothesis (19), This technique uses second antibodies conjugated with different chromophors and is now widely used for the study of the cellular colocalization of proteins with structural elements.

When cells were double stained with FTFC-stainine for vimentin (green) and with Texas red staining for hsp90 (red), followed by exposure of both staining patterns on the same film plaque, separate colored green and red filamen.s were shown (19). This was a clear indication that hcp90 does notcolocalize with vimentin. This finding was further supported by parallel experiments with cells treated with

triethyllead. A reorganization of vimentin filaments (green) became evident, without affecting the red hsp90 staining (19).

hsp90 CO LOCALIZATION WITH CYTQKERATIMS

• Using the above procedures of single and double staining immunofluorescence in Ishikawa ceils, it was demonstrated that hsp90 associates with cytokeratin intermediate filaments (19).

This became evident in cells treated with a mixture of cyiochalasin B and colchicine. Staining of the cells with anti-vimentin and anti-hsp90 antibodies offered figures with similarly reorganized filaments, showing an open lattice form including filament focal centers. This reorganization characteristics have been described for keratin cytoskeleton after combined treatment with these anticytoskeletal agents (36).

Double staining immunofluorescence microscopy in Triton-in soluble cytoskeletal preparations further corroborated this observation. By exposing both staining patterns on the same picture exclusively yellow filament structures were observed, when the cells were double stained for cy tokeratins with FITC-staining (green) and for hsp90 with Texas-red staining (red). This is shown in Fig. 3a, where the absence of any selectively green or red fluorescence is noticed.

Even more convenient, were the immunofluorescence staining patterns of the Triton-insoluble preparations of cells treated with the cytochalasin B/ colchicine mixture. Double stained and double exposed cells showed exclusively yellow colored filamentous structures which clearly demonstrated the well known reorganization characteristics of cytokeratin filaments (Fig. 3b).

a) b)

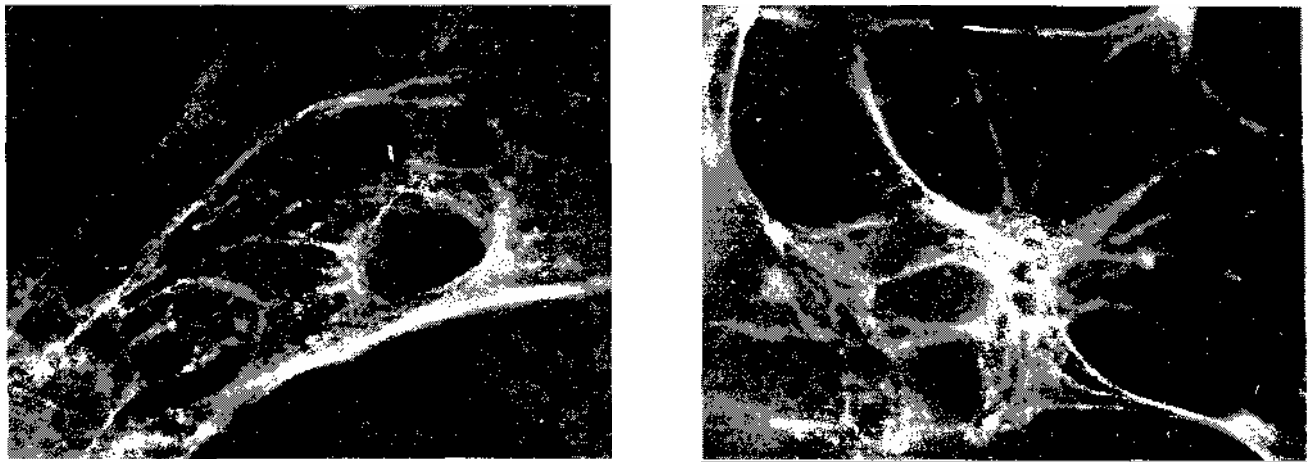

Figure 3. Double staining iinrnunof!.uorescence microscopy exposing both staining patterns on the same film plaque, (a) Triton-Insoluble cytoskeleton oflshikawa cells double stained for cy tokeratins (green) an:Shsp90(red). (b) Same as (a)but after confined treatment \\ithcytochalasinB and colchicine, showing the reorganization characteristics dt'Scribedforkeratincytoskeleton.Notethe exclusively yellow colored file, mntous structures and absence of any separate red or green fluorescence. (Experimental details in Kef 19).

SPECIFICITY OF THE AHTI-hsp90 AND ANTICYTOSKELETAL PROTEIN ANTIBODIES

• Since all data suinmari/.ed in this review deal with immunore act ions of cliiTerent anti-h^p90 and anti-cyloskeletal protein antibodies it was very imporrani 10 exclude any kind of cro^s reactions with investigated cytoskeletal proteins and cytosoiic preparations, respectively. For this purpose. Western blotting analyses ofcytosolic preparations were performed, using anti-hsp90. anti-tubulin. amivimentin and anti-cytokeratin antibodies. No eross reaction of anti-hsp90 antibodies with purified cytoskcletal proteins were observed. Moreover, ail

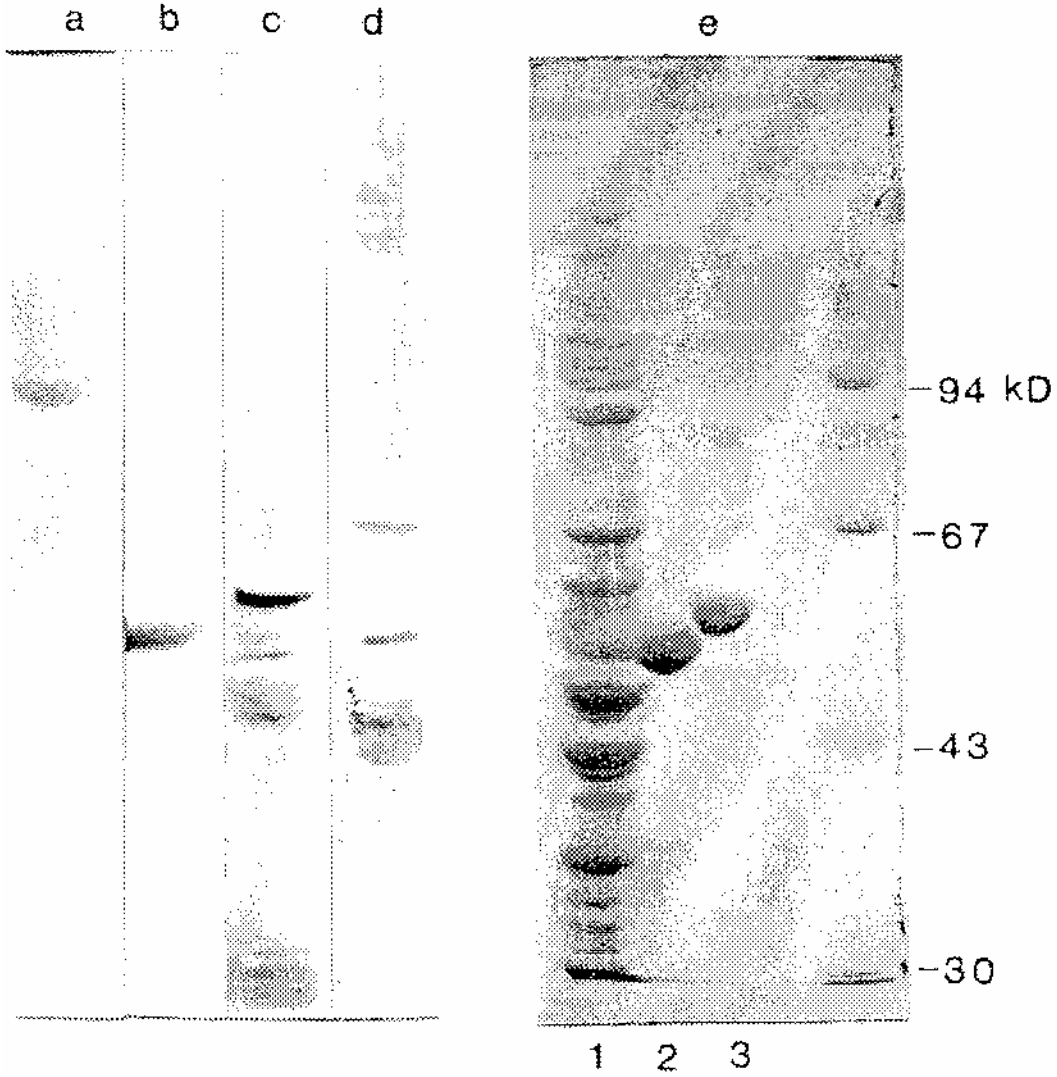

Figure 4. Western blonirtg analysis of cytosoiic preparations of Ishikcnva cells using ihe following aniihodiss: (a) ann'-hsp90, (b) atiii-tiibulin. (c) anti-viincntin, id) mui-cyiokemfms (8. S3). No <TO,S.V *reaction ofcmti-iisp90aniibodiescotdc!be visualized with cytosi)!icp*^{i}*~o!(?inN orpep'.iJefimigraiing toMW equal with lubulin. vhnetnin. orc\lakcninvs. (c) Couinassine bines!C'.inin?of(J) cytosolicpreparation of Jshika\va cells, (2) purified tttbiilin and (3) purified vmicn'ilin.*

anti-cytoskelelal antibodies did not cross-react with proteins in the range of 90 kD of the cylosolic preparations (Fig. 4). Thus the observed association ol hJpW \vithmicrotubulcs and cytokeraiins is not due to unspccific reaction of the anti-hsp90 antibody wiih these cytoskclctalsirucuires. Similar conclusions concerning the specificity of the anti-hsp90 antibodies were made also by Rcdmond el al (18).

BIOLOGICAL SIGNIFICANCE OF THE INTERACTION OF hsp90 WITH CYTOSKELETAL STRUCTURES

• Hsp90 is an ubiquitous, abundant and highly conserved protein performing essentialcellular functions. Its i nvolvcmcnt in transport processes in the cell has been speculated in the past (8). In the ca:;e of newly synthesi/ed pp60^{5K} and some other avian transforming virus proteinkinases, it was postulaled that hsp9() is implicated in their translocation from the site of synthesis to the plasma membrane (8.37,38). Moreover, in an earlier review dealing with the physiological role of heal shock proteins, the implication of hsp90 in ihe transport of steroid receptors to (he nucleus has been also discussed (7).

This hypothesis was further encouraged by the findings of the hsp90 colocali/aiion with microtubules (17-19) andcytokeratins (19) in intact cells, as well as by the association of cytosolic glucocortieoid receptor^ with cytoplasmic microtubules (39. 40). Recent lines of evidence suggest that cytoskeletal structures may also be involved in the intracellular protein transport (41). Thus, the colocalization of hsp90 with microtubules and cytokeratins and its association with the steroid hormone receplorrises ihe possibility that cytoskeletal structti res could be used as "rails" for the intracellular transport of steroid hormone receptors to the nucleus. This assumption is promoted by the colocalization of hsp90 with cytokeraiins, since microtubules do not have direct links with the nucleus, while the-intermediate filament protein lamin creates the nuclear lamina (42). Thus, we think that the colocalization of hsp90 with both cytoskelelal structures, the microtubules and the cytokeratin intermediate filaments, offer a well organized intracclkilar "highway[11] for (he cl'i-eci movement of the steroid hormone receptor via association-dissociation witli hsp90 molecules, from the cytoplasmic site of synthesis 10 the nuclear site of action. Once the "high way "is ready, the motors 143} are needed to ensure the transport on it. That may also be tested in the ca,^e ofhsp90-steroid hormone receptor translocaiion into the cell nucleus.

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