Evidence for reversible, non-microtubule and non-microfilament-dependent nuclear translocation of hsp90 after heat shock in human fibroblasts

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Fibroblasts—monoclonal antibodies—heat shock protein M_r 90000 (hsp90)—nuclear translocation—microtubules—microfilaments—confocal laser scanning microscopy—image analysis

A monoclonal antibody (29A) directed against rat liver heat shock protein M_r 90000 (hsp90) was produced. By Western immunoblotting of cytosols prepared from several different tissues and species, 29A was shown to specifically recognize only one band with $M_r \approx$ 90000.

Localization of hsp90 in human gingival fibroblasts was studied using the 29A antibody by indirect mono- and double-staining immunofluorescence and confocal laser scanning microscopy. The distribution was compared to that of the glucocorticoid receptor (GR) and various cytoskeletal structures.

Cells were analyzed in interphase and mitosis under basal culture conditions, after heat shock and after microtubule and microfilament depolymerization, sometimes combined with heat shock. A major part of hsp90 immunoreactivity was diffusely distributed throughout the interphase cytoplasm, but a weak nuclear staining with non-stained nucleoli was also present, however, only detectable after methanol and not after formaldehyde/Triton X-100 fixation. Heat shock induced a time-dependent translocation of hsp90 from the cytoplasm to the cell nucleus reaching a plateau after 15 h. This compartment shift was reversible and also occurred in the absence of intact microtubules or intact microfilaments.

Abbreviations. CLSM Confocal laser scanning microscopy.—ELISA Enzyme-linked immunosorbent assay.—FITC Fluorescein isothiocyanate.—GR Glucocorticoid receptor.—HRP Horseradish peroxidase.—hsp Heat-shock protein.—MTs Microtubules.—NC Nitrocellulose.—PBS Phosphate-buffered saline.—SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Introduction

The stress proteins or heat-shock proteins (hsps) include several proteins, usually classified in five major families according to their apparent M_r: hsp100/110, hsp90, hsp70, hsp60, and hsp10–30. They are expressed constitutively and/or after induction by stress. The hsps seem to form an important part of the cellular defense to various stressful events, such as heat, infection, radiation, hypoxia, inhibitors of oxidative phosphorylation, heavy metal ions, ethanol, certain chemicals and drugs (for a review, see [27]). It has been shown that after exposure to a stressing event, with subsequent transient or sustained increase of the cellular level of or change in the intracellular distribution of the stress proteins, the cell or organism acquires an increased resistance to either only the applied type of stressor or also various degree of cross-protection against other types of stressors as well [26, 43]. There are, however, many species and cell type-specific differences in stress response, and many agents only induce a selected subset of the hsps. The exact mechanism in which the stress proteins act to protect the cells is not known.

Furthermore, hsp60 and hsp70 act as dominant antigens in many infections with helminths, protozoa and bacteria [23, 28] and are induced during infection in both the invading microorganism and in the host phagocytes. Recently it has been suggested that stress proteins may serve as autoantigens involved in certain human diseases [23, 28].

Apart from the function of stress proteins indicated above, some of these proteins are expressed constitutively and may thus play an important role also under normal conditions. Heat-shock proteins like hsp70 and hsp60 seem to serve as "molecular chaperones" in the process of maintaining correct protein folding, transport and oligomer assembly [11, 16, 18].

Hsp90 constitutes 1 to 2% of the total cellular protein in many tissues [25], but the function of this protein has not

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yet been defined. A small amount of the cellular content of hsp90 has been reported to associate or interact with several intracellular proteins [17, 35, 38], among those actin [24] and steroid hormone aporeceptors [10, 15, 29, 37].

There is evidence for a developmental regulation of hsp90, independent from heat-shock induction [3], and hsp90 has also been shown to be a potent inhibitor of protein synthesis, possibly through activation of its associated enzyme eIF-2alpha-kinase [36]. Hsp90 has been shown to be identical with a tumor-specific transplantation antigen located on the surface of murine sarcoma cells [30, 41]. In yeast, two very similar hsp90 species have been observed, one of which is required for viability [27].

Hsp90 is required for steroid receptor action in vivo [33]. The role of hsp90 in relation to glucocorticoid receptors (GR) is dual. Hsp90 seems to confer to GR a conformation, which is necessary for high affinity ligand binding [6, 14], and hsp90 is also important in maintaining GR in a non-activated form, i.e., non-DNA-binding form [15]. Following steroid binding to the GR-hsp90 complex, hsp90 dissociates from the complex, and GR acquires specific affinity to glucocorticoid responsive elements in DNA [4].

The intracellular localization of hsp90 has previously been studied [5, 8, 12, 19, 24, 25, 34]. Hsp90 has been found to localize mainly in the cytoplasm, but has also been demonstrated in the nucleus. There is evidence that hsp90 interacts with various parts of the cytoskeleton, both the microtubules [34] and the microfilaments [24] (see also Discussion).

In this paper we report the production of a monoclonal antibody against hsp90 from rat liver. We have used this antibody in indirect immunofluorescence immunocytology to study the intracellular distribution of hsp90 in primary culture human gingival fibroblasts during basal culture conditions as well as after heat shock with or without intact microtubules (MTs) or microfilaments. The results were further analyzed by confocal laser scanning microscopy (CLSM) and image analysis.

Materials and methods

Production of a monoclonal antibody against rat liver hsp90

Immunization. Hsp90 was purified from rat liver cytosol by a two-step procedure as previously described [13]. BALB/c mice (Charles Rivers Inc., Norgate, Kent/UK) were immunized with rat hsp90. After a first subcutaneous (s.c.) injection of 50 µg hsp90 mixed with Freund's complete adjuvant (Difco Laboratories, Detroit, MI/USA), two subsequent s.c. injections of 50 µg hsp90 in Freund's incomplete adjuvant (Difco) were given with an interval of three weeks. Four days prior to fusion, daily intraperitoneal injections of 50 µg hsp90 were started and given on three consecutive days. Fusion was performed on the fourth day.

Cell fusion and cloning. Cell fusion was performed essentially as previously described [32]. Large scale production of monoclonal antibodies was performed by cultivation of the hybridomas in dialysis tubing in a continuously rotating cell culture flask according to a previously described procedure [39] in a Diacult system (Labassco, Partille/Sweden). Milligram amounts of antibody could be obtained in 1 to 2 weeks.

Immunoglobulin subclass determination. The immunoglobulin class and subclass of the obtained hybridomas was determined by enzyme-linked immunosorbent assay (ELISA) using goat anti-mouse immunoglobulin subclass antibodies (Southern Biotechnology Ass. Inc., Birmingham, AL/USA). These antibodies were conjugated with alkaline phosphatase.

ELISA screening to determine hsp90 reactive hybridomas. To screen for hybridomas producing antibodies against hsp90, an ELISA method was used. The wells of 96-well microtiter plates (Nunc, Copenhagen/Denmark) were coated with 100 ng partially purified hsp90 per well in 34 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6 at +4°C overnight. Non-specific binding to the plate was blocked by incubating the wells with 3% defatted milk in phosphate buffered saline (PBS) for 1 h at room temperature. Bound immunoglobulin from 50 µl hybridoma supernatant was detected by incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin diluted 1:400 (DAKO, Copenhagen/Denmark) for 1 h, followed by addition of enzyme substrate (0.55 mg/ml final concentration of 1,2-phenylene-diamine-dihydrochloride). Absorbance was determined with a TiterTek Multiscan MC-instrument (Flow Laboratories, Effab, Oy, Helsinki/Finland). Background values of absorbance were below 0.100. Supernatants were considered positive if absorbance values of > 0.500 were obtained on at least two different occasions.

SDS-PAGE and Western immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Bio-Rad Mini-PROTEAN cell, essentially as previously described [1], but using a 12% separating gel. A protein low molecular weight calibration kit used from Pharmacia, Uppsala/Sweden was used.

The 29A antibody was tested against purified hsp90. To ensure specificity of the antibody, it was also tested against crude cytosol from chick embryonal retina, rat liver, mouse liver or human leucocytes (obtained from plasmaferesis of patients with chronic lymphatic leukemia). As cross-reactivity of anti-hsp90 antibodies to the glucocorticoid receptor (GR) has been described previously [20], we extended specificity controls to include Western immunoblotting of purified GR probed with the anti-hsp90 antibody 29A. GR, purified by DNA- and DEAE-cellulose chromatography, as previously described [47], was a kind gift from Per-Erik Strömstedt.

Transfer of the proteins to nitrocellulose (NC) filters was performed as previously described [1]. The NC-filters were probed with antibodies against *a*) hsp90: protein A-purified 29A (see below), diluted 1:100 in PBS, (final protein concentration of 12 µg/ml), and *b*) GR: mab7 [32], produced as Diacult supernatants and purified by protein A, diluted 1:100 in PBS (final protein concentration of 10 µg/ml). Bound antibodies were detected by incubation with goat anti-mouse immunoglobulin conjugated to HRP diluted 1:3000 using 0.5 mg/ml final concentration of 4-alpha-chloro-1-naphtol as a substrate (both from Bio-Rad Laboratories, Richmond, CA/USA). Pre-blocking of strips and washes were performed as described above for ELISA.

Immunocytology

Cells and media. Cultures of normal human fibroblasts from a 12-year-old male and a 38-year-old male (both originally biopsy explants from the gingival mucosa) were subcultured as monolayers (5–20 passages) on glass coverslips at +37°C as previously described [1]. Cell media and supplementary components were purchased from Gibco, Uxbridge, Middlesex/UK.

Manipulation of cell cultures. In some experiments, cells were cultured in the presence of 10 µM colchicine, 10 µM vinblastine or 10 µM cytochalasin B (all from Sigma, St. Louis, MO/USA) for various times prior to fixation. For the heat shock experiments, the cells were exposed to +41 to +45°C for 1 to 25 h, as specified in

the Results section, and then fixed, either immediately, or after another 1 to 3.5 h recovery at +37 °C. In one set of experiments, the cells were exposed to a second heat shock at +45 °C for 1 h.

All incubations at the different temperatures were performed under otherwise identical moisture, CO_2 and pH-conditions as previously described [1]. In all heat-shock experiments, cells were transferred from a +37 °C incubator to a +42 °C incubator without prewarming the culture medium. Thus, the temperature of the culture medium was gradually increased to the desired level.

Immunostaining procedure. An indirect immunofluorescence mono- or double-staining technique [1] was used on cells grown on sterile glass coverslips. The fibroblasts were fixed in either *a*) cold methanol (−20 °C) for 10 min, or *b*) cold (+4 °C) 4% (w/v) formaldehyde in PBS for 10 min to 16 h followed by 0.05% (v/v) Triton X-100 (Sigma) for 30 min to 16 h after the respective pretreatments indicated above.

After washes, the cells were consecutively incubated with the first and second antibody (or mixtures of first and second antibodies for double-staining experiments) and mounted as described [1]. As fluorochromes we used fluorescein isothiocyanate (FITC) and Texas Red. These fluorochromes show a low amount of overlap [40] and can therefore be detected essentially independently of one another in the same cell. Microphotography was performed as described [1].

Confocal laser scanning microscopy (CLSM) and image analysis. In this study, a confocal laser scanning microscope developed at the Royal Institute of Technology, Stockholm/Sweden, [9, 49] was used to record approximately 1.0 µm thick transversal (= parallel to the plane of the coverslip), optical sections through the central part of the human gingival fibroblasts. For a description in more depth of the confocal laser scanning technique used in this study, see [9, 46, 49].

The data recorded by the microscope is stored as digital images where the value of each image element, lying between 0 to 255, is proportional to the fluorescent light emitted from corresponding points in the specimen. To calculate the relative intensity distribution in the cytoplasm and the cell nucleus in the same transversal, optical section through the central part of the cell, the image was shown on a TV-monitor, and the whole cell, the cell nucleus and a part of the background was encircled. The background that was due to autofluorescence from the specimen and also to an offset from the electronic part of the detector, was removed before the total intensities of the whole cell and the cell nucleus were calculated. The signal in the cytoplasm was then defined by subtracting the signal from the nucleus from that of whole cell. The mean intensity of each compartment for 10 to 14 representative cells for each experiment (see Results) was calculated, expressed in relative units and subjected to statistical analysis.

The inverted image on the TV-screen (Sun work station 386i) was photographed by a Pentax ASAHI (KX)-camera using a black-and-white 100 ASA film at an ASA setting of 100 and exposure time setting of 0.5 second.

Statistical analysis. A multiple comparison between the mean values of various treatments was made using Student's t-test. The levels of significance presented in the Results section have been adjusted according to the method of Bonferroni.

Antibodies. 1) The following primary antibodies were used: *a*) The above described Diacult-produced monoclonal mouse-anti-rat liver hsp90 IgG2a antibody, designated 29A, was purified on protein A Sepharose 4B (Pharmacia), eluted from the column with 0.1 M citrate buffer, pH 3.0, and subsequently dialyzed against PBS. The dialysate containing a total protein concentration of 1.2 mg/ml was diluted 1:25 in PBS, yielding a final protein concentration of 0.048 mg/ml. *b*) A monoclonal mouse-anti-rat liver glucocorticoid receptor IgG2a antibody designated "mab 7" [32] in ascites, diluted 1:50 in PBS, yielding a final protein concentration of 0.52 mg/ml. This antibody cross-reacts with the human GR [7]. *c*) A polyclonal rabbit anti-sea urchin egg tubulin heterodimer (Dakopatts, Glostrup/Denmark) in serum, diluted 1:100, yielding a final protein concentration of 0.51 mg/ml. *d*) A monoclonal mouse anti-chick brain alpha-tubulin IgG1 kappa (Amersham International, Amersham, Bucks./UK), in ascites diluted 1:100 in PBS yielding a final protein concentration of 0.05 mg/ml. *e*) A monoclonal mouse anti-chicken gizzard actin IgG1 kappa (Biogenex Lab., Dublin, CA/USA). *f*) Normal mouse serum.

2) The following secondary antibodies were used: *a*) An FITC-conjugated goat anti-mouse-Ig antibody (Becton Dickinson, Mountain View, CA/USA), diluted 1:10, yielding a final protein concentration of 0.025 mg/ml. *b*) A Texas Red-conjugated donkey anti-rabbit-Ig antibody (Amersham), diluted 1:25, yielding a final protein concentration of 0.020 mg/ml.

Results

Characterization of the anti-hsp90 antibody and specificity control

The 29A antibody belongs to subclass IgG2a and reacts well with hsp90 in ELISA, Western immunoblots and immunocytology. Figure 1a shows the Western immunoblot reactivity of antibody 29A with purified hsp90 as well as the lack of cross-reactivity with purified GR. Figure 1b shows that only one band around M_r 90000 is recognized by antibody 29A in crude cytosol from chicken retina, rat and mouse liver, and human leukemic lymphocytes.

Immunostainings of fibroblasts in interphase and mitosis comparing the distribution of hsp90 with that of putatively functionally related proteins

The monostaining pattern of hsp90 varied somewhat depending on the method of fixation/permeabilization of the cells (see Tab. I for a summary). Both fixation methods gave rise to a diffuse, predominantly cytoplasmic staining together with a weak, uniform nuclear signal with non-stained nucleoli (Fig. 2a). The nuclear hsp90 signal was stronger after methanol than after formaldehyde/Triton X-100 fixation, however always weaker than the cytoplasmic staining. Sometimes cells exhibited a more fibrillar hsp90 pattern in the peripheral parts of the cytoplasm, especially after formaldehyde/Triton X-100 fixation (not shown). Some parts of the plasma membrane, often the leading edge, were also usually stained.

Double-staining for hsp90 and tubulin usually showed different patterns as did staining for hsp90 and actin. Comparison of many cells monostained for either hsp90 or GR revealed that both these molecules were distributed in the cytoplasm, however, only GR exhibited a distinct fibrillar, usually MT-like, staining pattern as previously described [1].

We sometimes observed a weak hsp90 signal within the metaphase spindle (not shown). This was more often recognized after fixation with formaldehyde/Triton X-100 compared to methanol. During the other stages of mitosis, hsp90 was not seen to localize in the mitotic structures.

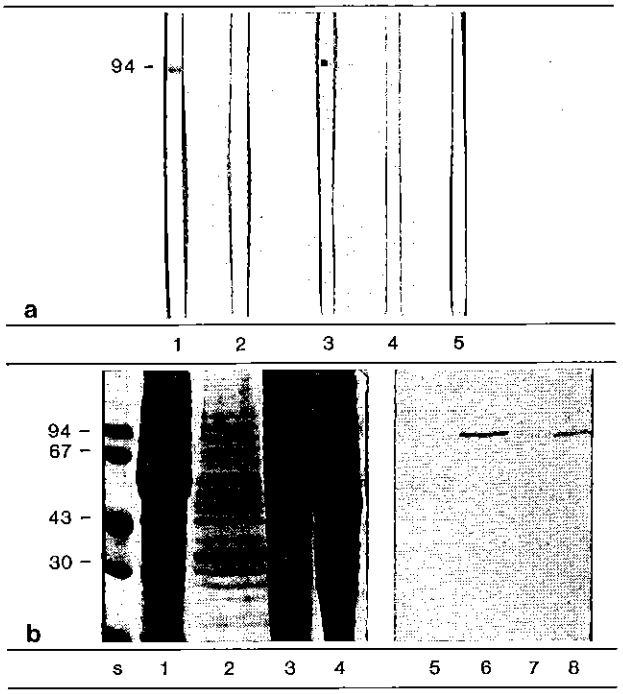

Fig. 1. Specificity of 29A anti-hsp90 and mab7 anti-GR antibodies in purified preparations (a) and in cytosolic preparations (b). — a. Purified rat liver hsp90 and GR were electrophoresed, electroblotted to NC-filter and subjected to Western immunoblotting. — *Lanes 1, 2*: purified rat liver hsp90 (5 µg/cm gel) probed with the monoclonal 29A anti-hsp90 antibody (*lane 1*) and normal mouse serum (*lane 2*). — *Lanes 3* to *5*: purified rat liver GR (0.5 µg/cm gel) probed with the monoclonal anti-GR antibody mab7 (*lane 3*), 29A (*lane 4*) and normal mouse serum (*lane 5*). — b. Crude cytosolic proteins from four species were electrophoresed (50–100 µg/lane). The proteins were Coomassie-stained (*lanes S, 1–4*) or electroblotted to NC-filter and probed with 29A anti-hsp90 monoclonal antibody (*lanes 5–8*). — *Lane S* contains the following standard proteins in M_r: phosphorylase B (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100). — *Lanes 1, 5*: embryonic chicken retina; *lanes 2, 6*: mouse liver; *lanes 3, 7*: human leukocytes; *lanes 4, 8*: rat liver. Molecular weight × 10^{-3} is indicated on the left.

Tab. I. Comparison of the immunolocalization of hsp90 in interphase and mitotic cells using two different methods for fixation/permeabilization.

	Methanol	Formaldehyde/ Triton X-100
Relative cellular distribution	c>n	c>n
Cytoplasmic pattern	diffuse, strong	sometimes fibrillar, strong
Nuclear pattern	diffuse, weak	very weak
Nucleolar staining	–	–
Plasma membrane staining	(+)	+
Mitotic metaphase spindle	–	(+)
Other mitotic stages	–	–
Increased nuclear staining after heat shock	yes	no

c Cytoplasmic. — n Nuclear. — +: positive. –: negative.

This was in contrast to tubulin and GR, which could be demonstrated to colocalize in the mitotic apparatus during all mitotic stages in most cells studied [1].

Effect of microtubule inhibitors on the immunostainings

Pretreatment of the cells with 10 µM colchicine or 10 µM vinblastine for 1 h prior to fixation has been shown to induce almost complete depolymerization of the cytoplasmic MTs in the human gingival fibroblasts used in this study [1]. After either of these pretreatments, hsp90 was found to be partly redistributed to the newly formed processes along the plasma membrane in the same manner as GR and tubulin [1]. The effect was similar for both colchicine and vinblastine and was the same whether the MT-depolymerizing agent had been present for one or 16 h. However, in contrast to GR and tubulin, hsp90 was not found in the vinblastine-induced inclusion bodies known as paracrystals.

Effect of heat shock on the immunostainings

The cells were heat-shocked using various temperatures, incubation time periods and recovery periods at +37 °C after heat shock, using either of the two fixation/permeabilization methods described above. After heat shock at +42 °C for 12 to 20 h followed by immediate fixation in cold methanol (−20 °C) for 10 min, there was a strong increase in nuclear hsp90 staining in virtually all interphase cells (cf. Figs. 2a and b). This finding was confirmed with two other primary cultures of human gingival and abdominal fibroblasts.

After heat shock, the cells were incubated at +37 °C for another 3.5 h, and the hsp90 immunoreactivity was found to return to the basal staining pattern. Using CLSM we observed this heat-induced reversible compartment shift also in 1 µm thin transversal, optical sections of the cells, thereby eliminating the possibility of projection artifacts (cf. Figs. 2c and d).

Heat shock at +42 °C with as short a duration as 1 h induced a similarly reversible pattern of nuclear hsp90 translocation, however, the nuclear staining was weaker and the finding less reproducible compared to the longer incubation times mentioned above. The maximal nuclear hsp90 staining intensity was found after around 15 h of heat shock at +42 °C. Prolonging the heat-shock period until 24 h did neither increase the overall or the nuclear hsp90 staining signal. These changes were detectable only after methanol fixation and were hardly observed after fixation with formaldehyde and Triton X-100 (not shown).

The heat shock-induced hsp90 response described above was also observed after continuous treatment of the fibroblasts with 10 µM colchicine, 10 µM vinblastine or 10 µM cytochalasin B during the 15 to 20 h heat-shock period, independent of whether MTs (using colchicine or vinblastine) or microfilaments (using cytochalasin B) were depolymerized starting 1 h before onset of heat shock or if depolymerization and heat shock were started simultaneously (not shown). Vinblastine-induced paracrystals did not contain hsp90 immunoreactivity, in contrast to what we have observed for GR and tubulin [1].

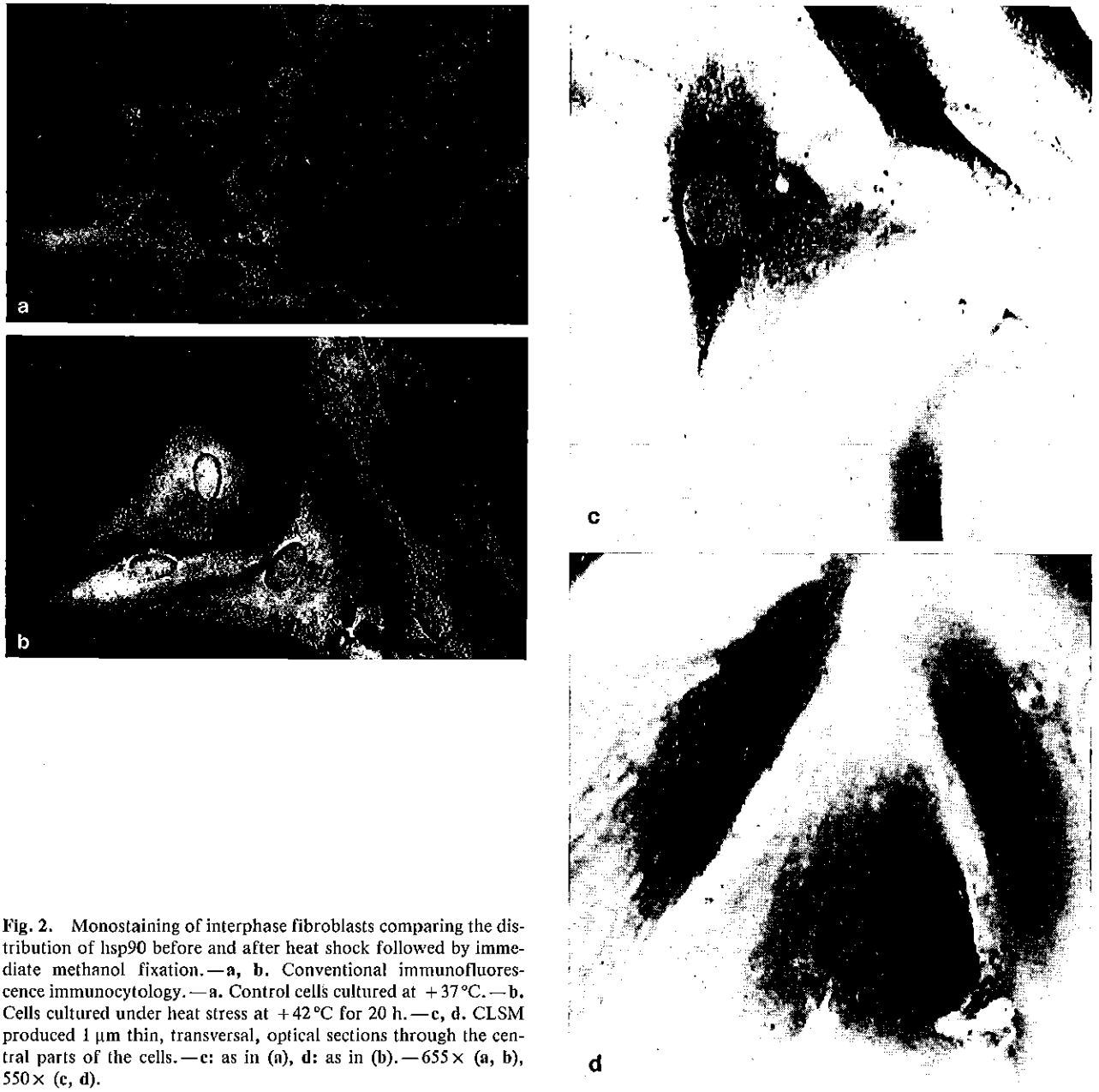

Fig. 2. Monostaining of interphase fibroblasts comparing the distribution of hsp90 before and after heat shock followed by immediate methanol fixation.—**a, b.** Conventional immunofluorescence immunocytology.—**a.** Control cells cultured at +37 °C.—**b.** Cells cultured under heat stress at +42 °C for 20 h.—**c, d.** CLSM produced 1 μm thin, transversal, optical sections through the central parts of the cells.—**c:** as in (a), **d:** as in (b).—655× (**a, b**), 550× (**c, d**).

By using CLSM, we quantified the changes in hsp90 immunostaining intensity on 1 μm thick transversal, optical sections after various pretreatments. A summary of these results is presented in Table II. During basal culture conditions, around 6 to 7% of the total hsp90 immunoreactivity was localized in the cell nucleus. Heat shock increased the relative nuclear hsp90 signal by ≈100% corresponding to around 13% of the total cellular signal, compared to the basal conditions ($p < 0.001$), while the total cellular hsp90 level did not show any significant changes (cf. Figs. 2c and d). Colchicine somewhat decreased the total intracellular hsp90 intensity, but simultaneous treatment with colchicine and heat shock still caused a similar relative increase in nuclear hsp90 signal, as was observed after heat shock alone ($p < 0.001$).

The fibrillar, MT-like, localization of GR [1] was not affected by +42 °C heat shock (not shown). Neither were the three cytoskeletal fibrillar networks of MTs, vimentin intermediate filaments and microfilaments affected by this level of heat shock. However, heat shock at +45 °C for 20 h caused breakdown of the structure of all three cytoskeletal systems: *a*) MTs: loss of fibrillar pattern, similar to the effect of colchicine; *b*) intermediate filaments: condensation around the nucleus, similar to the effect of colchi-

Tab. II. Subcellular distribution of hsp90, +/− heat shock, +/− MT depolymerization.

		Nucleus (N)		Cytoplasm (C)		N+C		N/(N+C)	
	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	10	0.13	0.02	1.86	0.35	1.99	0.35	6.7%	1.4
2	14	0.26	0.07	1.76	0.62	2.01	0.67	13.5%	3.6
3	11	0.10	0.01	1.78	0.52	1.88	0.52	5.7%	1.8
4	11	0.19	0.02	1.13	0.60	1.32	0.61	16.8%	5.7

1: Basal culture conditions at +37°C. — 2: Heat shock at +42°C 20 h. — 3: As in (2), followed by recovery as in (1) for 3 h. — 4: Colchicine-induced MT depolymerization for 1 h before onset of heat shock as in (2) in continuous presence of colchicine. — N Signal intensity in the nucleus. — C Signal intensity in the cytoplasm. — N+C Total signal intensity in the whole cell. — N/(N+C) Quotient (expressed as percent) between the signal intensity in the nucleus versus that in the whole cell. — Relative distribution of hsp90 immunofluorescence intensity between the nuclear (N) and Cytoplasmic (C) compartments in 1 µm thin optical, transversal sections obtained by CLSM. After various pretreatments followed by methanol fixation, the fibroblasts were sectioned through their central parts using identical settings of the confocal laser scanning microscope. — The values are expressed in relative, arbitrary units, which are proportional to the intensity of the fluorescent light signal reaching the detector of the microscope. Each value represents the mean value of the hsp90 signal intensity in optical sections from the indicated number (n) of fibroblasts. — SD Standard deviation.

cine; *c*) microfilaments: an "arborization pattern", similar to the effect of cytochalasin B (not shown) together with the formation of actin-containing nuclear inclusion bodies [44].

A second 1 h heat shock at +45°C after 8 h +37°C recovery following a 20 h primary +42°C heat shock showed a similar disintegration of the above mentioned three parts of the cytoskeleton as a 20 h primary heat shock at +45°C. After this level of heat stress, hsp90 exhibited essentially the same staining signal as during basal culture conditions, i. e., the second short heat shock did not induce a visible nuclear translocation of hsp90.

Discussion

During basal culture conditions, we observed a strong, mainly diffuse cytoplasmic and much weaker nuclear hsp90 staining in interphase fibroblasts. Heat shock induced a time-dependent translocation of hsp90 from the cytoplasm to the cell nucleus reaching a plateau after 15 to 20 h, when the nuclear hsp90 level had roughly doubled. This process was reversible upon reincubation at normal temperature. The level of heat shock was chosen to mimic the temperature that human cells might be exposed to during severe clinical fever conditions, both regarding intensity and duration.

Colchicine causes some reduction in total cellular hsp90 level, probably due to a reduction of protein synthesis by the used concentration of colchicine [48]. However, heat shock following previous MT and microfilament depolymerization with colchicine, vinblastine or cytochalasin B present throughout the entire heat-shock period still caused a significant nuclear translocation of hsp90. This strongly indicates that the compartment shift does not require intact MTs or microfilaments and must be mediated through another transport mechanism. These findings are in line with the results of Welch et al. [45] who found that the heat-induced (+42°C, 4 h) transport of hsp72 from the cytoplasm to the nucleolus was independent of an intact cytoskeleton.

There are several previous reports describing the localization of homologous proteins belonging to the hsp90 family in cells or tissues from various species using different antibodies and different fixation/permeabilization techniques. Our data are in accordance with some studies, but not with others.

Some authors [5, 8, 12, 19, 25, 42] report a mainly diffuse cytoplasmic and/or nuclear staining whereas others [24, 34] have found a more structured intracellular distribution.

Van Bergen en Henegouwen et al. [42] and Berbers et al. [5] used an affinity-purified rabbit anti-mouse neuroblastoma hsp84 antibody as well as monoclonal anti-hsp84 antibodies and found hsp84 to be evenly distributed throughout the cytoplasm and also in the cell nucleus in five different mammalian cell lines. Heat shock at +42°C for 4 h induced a stronger nuclear staining relative to the cytoplasmic staining, and this change was reversible upon reincubation at +37°C. One of their cell lines, a mouse fibroblast cell line, exhibited a basal hsp84 distribution and heat shock-induced change in hsp84 localization that was quite similar to our results obtained in human gingival primary culture fibroblasts. However, the other three of their four cell lines exhibited a strong nuclear staining already under basal culture conditions. It may be noted that these three cell lines were derived from the ectodermal and entodermal embryonic germinal layers, as opposed to the mesodermically derived fibroblasts. Thus, there may be a germ layer-related difference in the cellular distribution of hsp90.

Carbajal et al. [8] produced an affinity-purified rabbit anti-Drosophila hsp83 antibody and found the hsp83 immunofluorescence in Drosophila melanogaster Kc-cells to be distributed diffusely throughout the whole cytoplasm with nonstained nuclei. Heat shock at +37°C for 1 to 2 h did not increase the nuclear staining.

In immunohistological studies using a monoclonal mouse anti-Achlya ambisexualis hsp88 antibody (AC88), Gasc et al. [19] found hsp88 to be located both in the cytoplasm and in the cell nuclei in paraffin embedded as well as in frozen sections of rabbit uteri. Lai et al. [25] produced a monoclonal rat anti-human testis hsp85 antibody which located the hsp85 immunoperoxidase signal in HeLa cells exclusively to the cytoplasm.

Collier et al. [12] found that hsp89 in non-stressed chick fibroblasts exhibited a uniform cellular staining pattern

with some concentration in the nuclear and perinuclear area. Heat shock at +45 °C for 3 h induced an increased hsp89 signal throughout the cell that was reversible upon recovery at +37 °C. A second heat shock at +45 °C for 3 h, however, caused a strong and uniform nuclear accumulation of hsp89 along with a reduction of the cytoplasmic hsp89 signal.

There are several reports indicating an interaction or colocalization between various heat-shock proteins and cytoskeletal proteins. Koyasu et al. [24] provided evidence for two mammalian heat-shock proteins (hsp90 and hsp100) to be actin-binding proteins in a human epidermoid carcinoma cell line. Double-staining experiments indicated a colocalization of hsp90 and filamentous actin in membrane ruffles. The same group, Nishida et al. [31] presented data indicating that both hsp90 and hsp100 are bound to actin in a calcium-dependent fashion; i. e., calmodulin and calcium prevent binding between the hsps and actin. However, in contrast to this, we did not find any colocalization of hsp90 and the intracellular microfilament (actin) pattern.

Furthermore, we have not been able to convincingly reproduce the results of Redmond et al. [34], who found that hsp88 is colocalized with cytoplasmic MTs in six different mammalian cell lines including human HeLa cells, using the AC88 antibody. Our data indicate that there seems to be some MT-like hsp90 staining pattern after aldehyde/detergent fixation in interphase fibroblasts, that is extracted by alcohol during sample processing. There is a similar redistribution of hsp90 as of tubulin and GR to newly formed plasma membrane processes along the cellular periphery after artifically induced MT depolymerization, regardless of which of the two fixation techniques. This redistribution does not occur for actin or vimentin. However, our findings of hsp90 being *a*) mainly diffusely localized in the cytoplasm, *b*) absent from the mitotic apparatus during most stages of mitosis, and *c*) absent from vinblastine-induced paracrystals, argue against a structural interaction between hsp90 and MTs.

The anti-hsp88 AC88 antibody has been shown to cross-react with rodent GR [20]. Other groups have not observed this non-specific reactivity [34]. The antibody 29A reported in this study shows no cross-reactivity with GR. However, some of the monoclonal antibodies against hsp90 that we raised simultaneously with 29A showed cross-reactivity with GR. This indicates epitope similarities between these two proteins and may give rise to problems in immunocytological and immunochemical studies.

The two different fixation/permeabilization techniques in our study gave rise to somewhat different hsp90 staining patterns, summarized in Table I. Several explanations for the differences are possible: (*i*) aldehyde/detergent did not allow access of the hsp90 antibody to its antigenic epitopes in the nuclear compartment, (*ii*) detergent could have extracted loosely bound nuclear hsp90, or (*iii*) alcohol may have extracted parts of hsp90 bound to membranes, the mitotic spindle and possibly MTs. It is important to point out that the heat-induced effects on hsp90 were only detectable after alcohol fixation. A similar fixation dependence in the localization of hsp70 has been reported by Collier [12].

If only a minor portion of hsp90 is localized in the nucleus, this may be obscured by a strong immunofluorescence signal from the cytoplasm. By using CLSM we produced 1 µm thin optical sections of cells, thereby eliminating the possibility of projection artifacts. Our results show that a minor portion (≈ 7%) of the total cellular content of hsp90 is located in the nucleus under basal culture conditions. An exclusively cytoplasmic localization of hsp90 in the non-stressed cell is not consistent with the alleged interaction and functional importance of hsp90 in relation to the sex steroid receptors, i. e., the estrogen receptor and progestin receptor, which have been reported to be localized mainly in the cell nucleus [22].

From Table II it is evident that the total signal intensity in the cell was the same before and after heat shock with or without recovery at +37 °C. The hsp90 level in the nucleus was measured in CLSM optical sections to be increased by around 100% ($p < 0.001$) and since this increment was not due to a measurable increase in the total cell hsp90 intensity, the data indicate that this level of heat shock induces mainly a translocation of hsp90 from the cytoplasm to the nucleus and is not due to heat-induced de novo synthesis of hsp90. This finding is in line with the results of Berbers et al. [5].

The possible physiological implication of translocation of a pool of hsp90 to the cell nucleus after heat shock is presently unclear. The results presented here indicate that the cell has access to a nuclear translocation mechanism for hsp90 that does not operate through any of the three main cytoskeletal networks. This putative transport machinery must have the capability to resist heat shock and may be vital for the survival of cells during severe stress conditions, such as heat shock.

Since it has been shown that heat shock causes a general inhibition of gene transcription, besides a specific increased transcription of a small number of heat-shock protein genes [2, 27], one may speculate that a portion of the hsp90 pool moves to the nucleus to participate in a general down-regulation of gene expression, acting as a direct or indirect negative transcription factor, while the transcription of specific heat-shock genes is stimulated through specific heat-shock factors. Some evidence in favor of this possibility has been presented [2].

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