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Cell stress induces association of heat shock proteins with the cytoskeleton

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The association of heat shock proteins (HSPs) with the cytoskeleton is important, since the actin-based cytoskeleton is a sensitive monitor of extra cellular stimuli and is critical in healing. Regarding HSPs, they operate with a number of homeostatic mechanisms to maintain the cellular functions during and after stress. Current investigation assesses the interaction of the heat shock proteins with the cytoskeleton proteins. For this purpose, HEp-2 cells were stressed by UVA or by caloric irradiation. The expression of HSP and cytoskeleton proteins was monitored by immunofluorescence and Western blot with monoclonal antibodies directed against HSP60, HSP70, HSP90, actin, tubulin and cytokeratin and polyclonal anti-HSP27. As expected, UVA irradiation and heat induced a ubiquitous expression of HSP, detected as sparse granules in the nucleus, the cytoplasm and the perinuclear area; HSP90 was chiefly expressed along the cytoskeleton, and was associated with actin and cytokeratin. Finally, the stress triggers HSP response, which is involved in cytoskeleton modification.

Key words: UVA irradiation, HSP, caloric stress, cytoskeleton.

INTRODUCTION

The skin is a protective barrier targeted by environmental stressors like the sun light, which contains a broad spectrum of UV light including UVB (290-320 nm) and UVA (320-400 nm) spectra, both inducing photo-damage. Epithelial lesion is accompanied by IL-1, IL-6 and TNFa production that in turn induces inflammation (Yano *et al*., 2008). After sun irradiation, the epithelia trigger different homeostatic mechanisms of repair, which are mediated by heat shock proteins (HSP). These mechanisms determine cell death or survival (Lowe *et al*., 1995; Leverkus *et al*., 1998; Avalos-Diaz *et al*., 1999; Kwon *et al*., 2002; Nadeau & Landry, 2007).

Six main families of mammalian HSP have been described so far. The possible interaction between HSPs and the skin has been extensively studied; such relationship is important in healing. For instance, HSP27 stabilizes the stress filaments during oxidative stress and regulates the keratinocyte differentiation (Jonak *et al*., 2002), HSP60 induces keratinocyte proliferation and migration (Zhang *et al*., 2004), HSP70 is a chaperone related to protein synthesis and keratinocyte growth (Gething & Sambrook, 1992), Hsp90 is associated with epidermal differentiation, while HSP110 induces thermo-resistance and possesses chaperoning activity (Laplante *et al*., 1998).

The actin-based cytoskeleton is a sensitive monitor of extra cellular stimuli and is also critical in healing (Liu & Fletcher, 2006). Based on this point, it would be interesting to assess the relationship between HSP and cytoskeleton proteins under stress. In the present studies HEp-2 cells were stressed by caloric and UV irradiation and the interaction between HSPs and the cytoskeleton was addressed.

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MATERIALS AND METHODS

Cell culture

The human epithelial cell line HEp-2 cells (ATTC) were grown at 37°C, in an atmosphere of 5% CO_2, in DMEM media (Sigma St. Louis, Mo.) supplemented with penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), insulin (0.08 U ml^{-1}), and 10% foetal bovine serum (FBS) (Gibco BRL, Grand Island, NY). Cells growing in logarithmic phase were harvested by means of trypsin EDTA and plated at confluence for experiments with stressors (5×10^6 cells ml^{-1}).

Cell stressors

Cultures were irradiated at 366 nm using a UVA lamp (Black-Ray lamp UVL-56), achieving a dose of 5-30 mJ cm^{-2} (Leverkus *et al*., 1998). Control cells were cultured and manipulated without irradiation. Caloric stress was done by raising the temperature of the cell cultures from 37°C to 40°C, 42°C and 44°C respectively, for a duration of 3 hrs.

Metabolic radio labelling

Cell cultures were pulsed with 10 μCi of ^{35}S-methionine (Amersham). Pulsing was applied at the end of the stress. Afterwards, cultures were washed and normal media without ^{35}S-methionine were incorporated into the cultures. Finally, cells were harvested and radiolabelled extracts were obtained as described further on.

Protein extraction

After stress, cells were washed in PBS, and lysed in 1% Triton X-100, 140 mM NaCl, 1 mM EDTA, 10 mM Tris-HCI pH 7.6 and 1 mM PMSF. Cell extracts were homogenized and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were collected and probed. The cytoskeleton fraction was solubilized by 30 min incubation in urea buffer (1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM PMSF, in 10 mM Tris-HCl and pH 7.6 with 8 M urea). Protein concentration was measured by the Bradford method. The supernatants and pellets were boiled for 5 min in the sample buffer and electrophoresed onto 10% polyacrylamide-SDS gels (Laemmli, 1970). Because cell extracts were divided into soluble and insoluble fractions, the total amount of protein tested resulted from the addition of both fractions.

Protein blotting

SDS-PAGE gels were transferred using Hybond-C nitrocellulose sheets (Amersham, UK) and applying the method of Towbin *et al.* (1979). Immuno-reactive bands were identified with a 1:1000 dilution of monoclonal anti-HSP60, anti-HSP70, anit-HSP90, anti-actin, anti-tubulin and anti-cytokeratin antibodies and with the polyclonal anti-HSP27. Nitrocellulose sheets were incubated for 1 hr with the second antibody peroxidase-conjugated goat anti-mouse IgG (H-9044, Sigma), and peroxidase-conjugated goat anti rabbit (A-9169, Sigma) for HSP27. Immuno-reactive bands were detected by chemiluminescence (ECL, RPN2106; Amersham, UK).

Radio-immunoprecipitation assay

Radiolabeled antigens were immunoprecipitated with anti-HSP70 linked to Sepharose-SPA. Immunoadsorbents were prepared by linking the antibodies to Sepharose 4B-SPA (Sigma, St Louis Mo) dissolved in 50 mM Tris, pH 7.0. The coupling was achieved by rocking the mixture 3 hrs at 4°C, the immunoadsorbent was extensively washed with 0.15 M Tris, pH 7.5. Radiolabeled antigens and immunoadsorbent were mixed, incubated and rocked at room temperature overnight. After extensive washings, immunoprecipitates were eluted with 0.1 M glycine at pH 3.0, followed by neutralization with 1 M Tris-base, pH 9.0 and characterized by SDS-PAGE and autoradiography.

Co-precipitation assay

Unlabeled cell extracts were co-precipitated with anti-cytokeratin, tubulin or actin antibodies linked to Sepharose-SPA. Antigens and immunoadsorbent were mixed, incubated and rocked at room temperature overnight. After extensive washings; immunoprecipitates were eluted with 0.1 M glycine at pH 3.0, followed by neutralization with 1 M Tris-base, pH 9.0. Then, the immunoprecipitates were then electrophoresed, blotted and tagged with anti-HSP27, anti-60, anti-70 or anti-HSP90 or *vice versa* and immuno-reactive bands were detected by chemiluminescence.

Double fluorescent labelling

Co-localization of HSP and cytoskeleton-associated proteins was studied by double labelling as follows: a first incubation with anti-anti-HSP27, HSP60, HSP70, HSP90 antibodies was followed by 1 hr incubation with sheep anti-mouse or goat anti-rabbit FITC label-

led antibody (Cappel, West Chester, PA). After extensive washings, the slides were incubated with anti-actin, b-tubulin and cytokeratin antibodies tagged in red by rodamine (Sigma). Finally, the slides were washed, mounted and examined under a confocal scanning microscope LSM (Axiovert 200M, Carl Zeiss, Göttingen Germany). Fluorescein and rodamine filter combinations with excitations of 450-490 nm and emissions of 515-565 nm were used respectively. Objectives were LCI "Plan-Neofluar" and image processing was done with a Zeiss LSM Image Examiner.

Antibodies

The following polyclonal and monoclonal antibodies were used: rabbit polyclonal IgG HSP27 (H-77, Sc-9012, Santa Cruz Biotechnology), monoclonal anti-HSP60 (H-4149), anti-HSP70 (H-5147), anti-HSP90 (H-1775), anti-actin (A-4700), anti-b-tubulin (T-5168) and anti-cytokeratin (C-6909) (Sigma, St Louis MO).

Statistical analysis

The densitometry value of chemiluminescence from autoradiography was recorded, and the intensity of the bands was quantified by an image processor (Bio-Rad GS-670). Data were expressed in arbitrary densitometry units and processed by Student's t-test (Prisma program); statistical significance level was set to $p < 0.005$.

RESULTS AND DISCUSSION

Stress increases HSP response

HSPs were broadly expressed in the soluble and insoluble fractions of control cells. However, at 37˚C, HSP60 and HSP70 were better detected than other HSPs. As anticipated, under stress, there was a significant increase of the soluble fractions of HSP27, HSP60 and HSP70 (Fig. 1 and Table 1).

Stress triggers de novo synthesis of HSP

To address the question whether HSP increase was due to a *de novo* synthesis, a metabolic radiolabelling with ^{35}S-methionin and immunoprecipitation assays with anti-HSP70 was carried out. The results of this experiment showed that the increase of HSPs during stress was the result of *de novo* synthesis (Fig. 2).

Stress redistributes HSPs

Since HSP60, HSP70 and HSP90 expression is constitutive, all of them were faintly detected as sparse

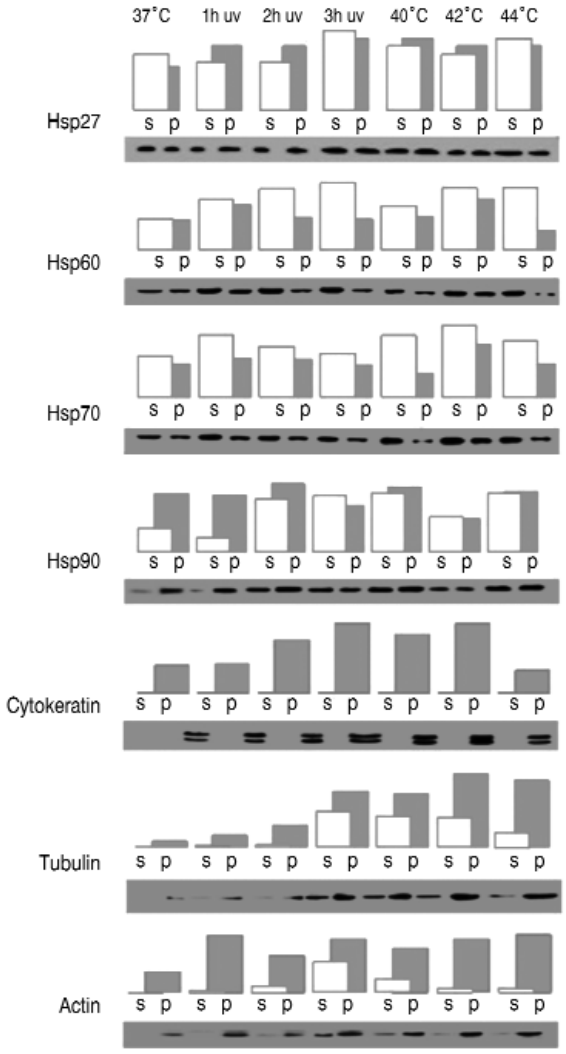

FIG. 1. Western blot analysis from cytosolic (s) and cytoskeletal (p) fractions of stressed HEp-2 cells by UVA and heat. Vertical bar graphics correspond to the sum of intensities recorded by densitometry from each band.

granules within the nucleus and the cytoplasm of non-stressed HEp-2 cells. However, stress induced the following modification in the fluorescent pattern: HSP were relocalized at the perinuclear domain. In the case of HSP70, this was polarized and distributed along the Golgi compartment, forming aggregates. Surprisingly, HSP27 expression was faint (Fig. 3).

Stress modifies microtubule and microfilament distribution

We speculate that actin, tubulin and cytokeratin were modified under stress. Our results showed that these proteins were detectable in the insoluble fraction of control cells as demonstrated by immunoblotting assays. Nevertheless, under stress, actin and tubulin

TABLE 1. Densitometric values (mean ± s.d.) by Western blot

	Control	1hUV	2hUV	3hUV	40˚C	42˚C	44˚C
Hsp27-s	38853 ± 11.8	30175 ± 2.8*	33708 ± 3.2	49358 ± 3.2*	42566 ± 2.7*	36232 ± 3.6	44358 ± 3.0*
Hsp27-p	32377 ± 3.8	39155 ± 2.4*	39668 ± 2.7*	47031 ± 3.0*	45046 ± 2.8*	37626 ± 3.7	39702 ± 3.0*
Hsp60-s	20029 ± 26.0	32275 ± 14.0	38918 ± 18.0	42664 ± 16.0*	42653 ± 6.0*	39491 ± 4.0*	40103 ± 8.0*
Hsp60-p	19058 ± 4.0	28515 ± 2.0	20254 ± 4.0	19371 ± 4.0	21383 ± 4.0	32178 ± 2.0*	12004 ± 3.0
Hsp70-s	28934 ± 4.0	44090 ± 8.0*	36307 ± 4.0*	31236 ± 4.0	44131 ± 5.0*	50924 ± 3.0*	39938 ± 7.0*
Hsp70-p	23139 ± 6.0	27951 ± 5.0	26783 ± 4.0	22445 ± 3.0	17016 ± 3.0	35345 ± 3.0*	23392 ± 4.0
Hsp90-s	14772 ± 4.0	8968 ± 2.0	33665 ± 3.8*	35514 ± 4.0*	37312 ± 3.0*	22018 ± 6.0	37416 ± 2.0*
Hsp90-p	36545 ± 3.0	35702. ± 4.0	42839 ± 1.7*	29110 ± 1.8	40908 ± 4.0	20895 ± 3.0	38093. ± 4.0
Cytokeratin-s	13.6 ± 3.0	13.6 ± 3.0	13.6 ± 3.0	13.6 ± 3.0	13.6 ± 3.0	13.6 ± 3.0	13.6 ± 3.0
Cytokeratin-p	24384 ± 9.5	26217 ± 1.8	46792 ± 3.7*	62080 ± 3.4*	51831 ± 3.5*	61928 ± 4.0*	19874 ± 3.4
Tubulin-s	9.2 ± 1.2	705 ± 3.0*	954 ± 3.9*	17711 ± 7.0*	15510 ± 4.2*	14757 ± 4.2*	7996 ± 3.7*
Tubulin-p	2986 ± 2.7	5626 ± 4.0*	10817 ± 2.8*	27911 ± 6.9*	26706 ± 5.4	36893 ± 3.9*	33662 ± 3.1*
Actin-s	9.2 ± 1.2	532 ± 1.2*	2562 ± 2.9*	12854 ± 4.4*	5442 ± 2.9*	1941 ± 2.2*	1702 ± 2.5*
Actin-p	8750 ± 1.8	24003 ± 3.4*	15432 ± 3.6*	22393 ± 3.3*	18672 ± 2.5*	22671 ± 2.2*	24211 ± 2.2*

s = cytosolic fraction, p = cytoskeletal fraction

* statistically significant < 0.005 by t test

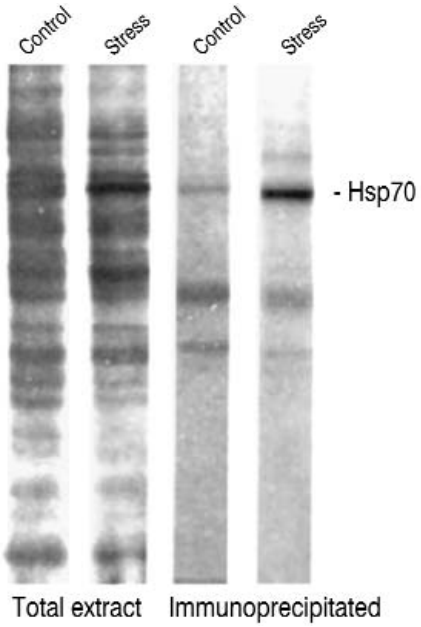

FIG. 2. Immunoprecipitation of radio-labelled ^{35}S-methionin-Hsp70 *de novo* synthesized under stress.

were detectable in soluble fraction, and this finding suggests that stress destabilizes microtubules and actin-based microfilaments. An interesting observation was the lack of soluble cytokeratin, which suggests that the intermediate filament-dependent structure is not modified by stress. Incidentally, actin-dependent fibres were rearranged as multiple stress fibres, as immunofluorescent assays revealed. Interestingly, under stress, the cytoskeleton-associated proteins increased by 3-fold (Fig. 1).

HSPs interact with cytoskeleton proteins

The possible interaction between HSPs and cytoskeleton-associated proteins was explored by double fluorescent labelling assays, with the following results: HSP60 and HSP70 were co-localized with actin and/or tubulin, and faintly with cytokeratin (Fig. 4). This suggests a possible role of the HSP proteins in cytoskeleton reorganization, especially for the actin-based stress fibres. The above association was further characterized by co-immunoprecipitation with anti-cytokeratin, tubulin or actin antibodies linked to Sepharose-SPA. The immunoprecipitates were then electrophoresed, blotted and tagged with anti-HSP27, anti-60, anti-70 or anti-HSP90 or *vice versa*. The results of these experiments showed an interaction bet-

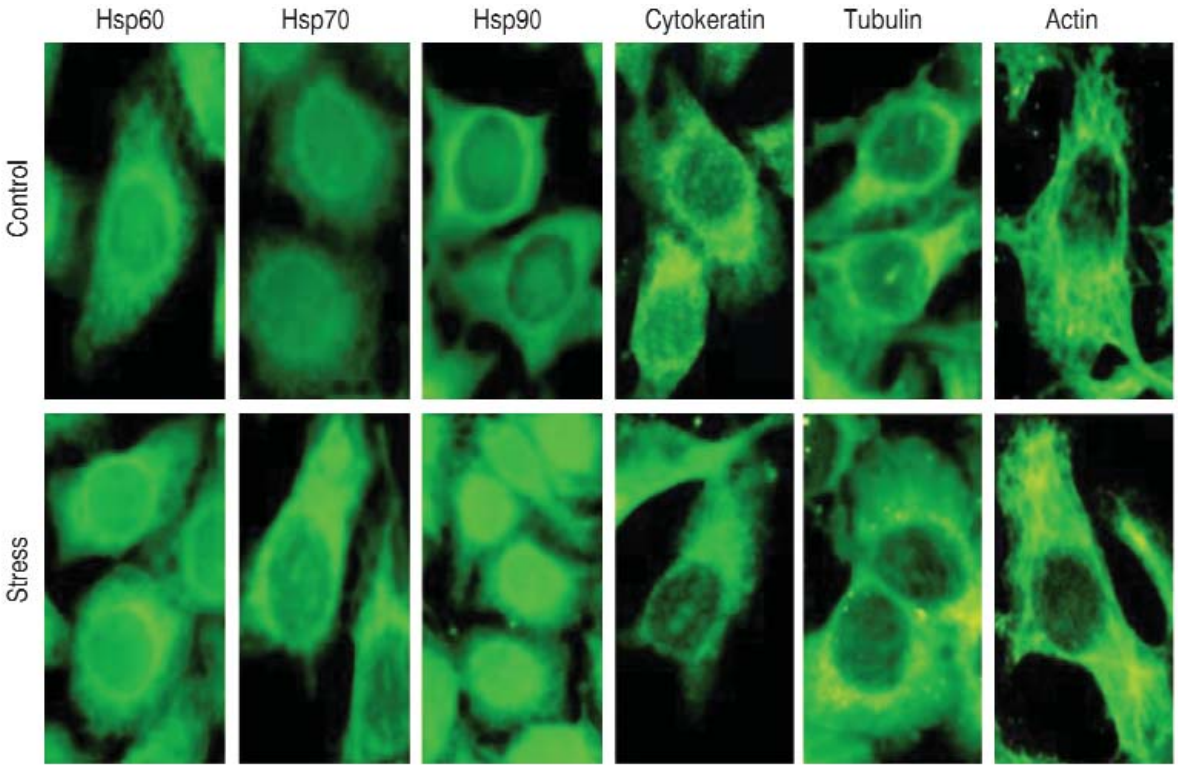

FIG. 3. A representative immunofluorescence of UV stressed and control HEp-2 cells tagged with monoclonal antibodies against HSPs or against cytoskeletal components.

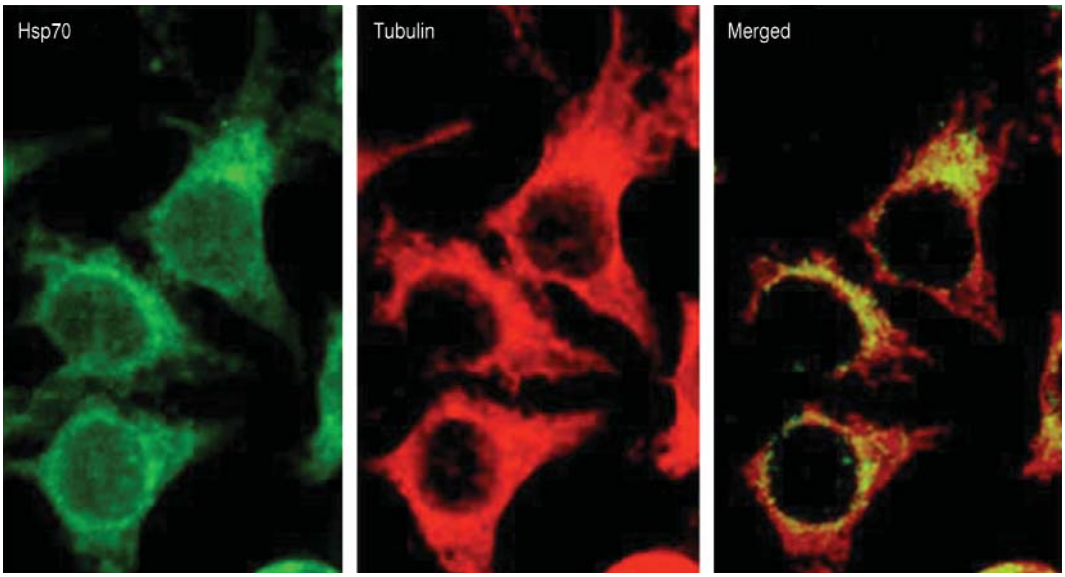

FIG. 4. Co-localization of HSP70 with tubulin, and the merge in yellow.

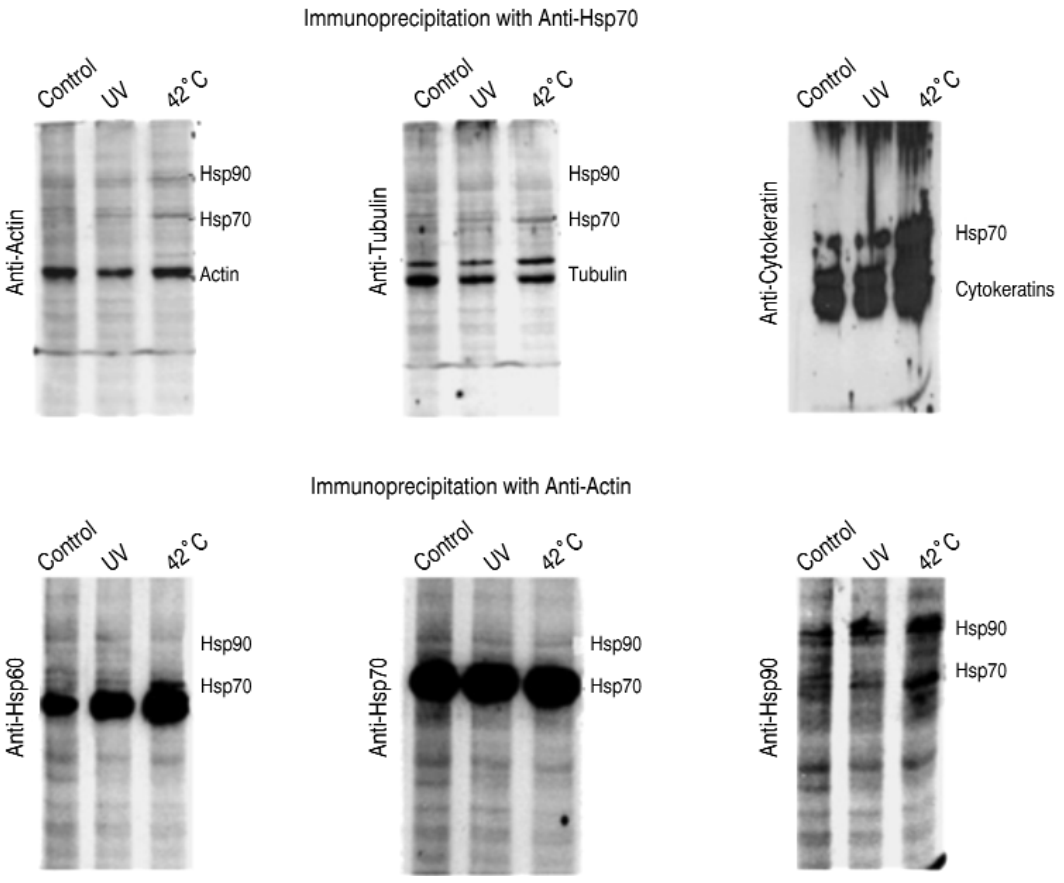

FIG. 5. Crossed-immunoprecipitation. The top of each panel corresponds to the monoclonal used to immunoprecipitate the cell extract. On the left, the monoclonal used to disclose the co-precipitated proteins.

TABLE 2. Interaction between HSPs and cytoskeleton proteins assessed by crossed-immunoprecipitation

Immunoprecipitation by	**anti-Actin**	**anti-Tubulin**	**anti-Cytokeratin**
Interaction with	HSP27, HSP70, HSP90	HSP27, HSP60, HSP70, HSP90	HSP70

ween actin or tubulin and HSP60 or HSP70 (Fig. 5 and Table 2).

Current studies address the issue whether heat shock proteins play a role in cytoskeleton modification. The main results of ongoing investigations are the following: i) HSPs are broadly distributed within the cell compartments, ii) HSPs increase under stress, and iii) stress induces changes in the cytoskeleton and association with HSPs. Such association is probably related to microtubule and microfilament rearrangement.

HSP and actin-based filaments play a significant role in the response to external stimuli (Guay *et al*., 1997). However, during cell stress, an interaction between HSPs and actin is set up, but the purpose of such an interaction is still unclear. It is well known that stress triggers HSPs, but simultaneously these proteins participate in the rearrangement of the actin-based microfilaments. HSPs probably avoid abnormal filament-filament interaction and may prevent incestuous cross-linking between filaments or between filaments and proteins (Fuchs & Cleveland, 1998).

Stress by heavy metal microtubule poisoning, indicate that kinesine-like proteins are mediators of depolymerization; zinc-induced tubulin polymers are characterized by an anti-parallel protofilament arrangement, the microtubule depolymerization is induced by XKCM1, which is a kinesin-like protein (Niederstrasser *et al*., 2002). Kinesins are proteins that use the energy of ATP to translocate along microtubules. Some kinesin-like proteins destabilize the microtubule ends, and are called as "catastrophe kinesins" or MCAK/XKCM, this notion is essential for understanding how microtubules in a cell are re-arranged during stress (Kinoshita *et al*., 2006).

In current results, the presence of HSP90 in the cytoskeleton and its virtual absence in the cytosol of normal cells, suggest that HSP90 may contribute to cytoskeleton organization in the absence of an external stimulus. Furthermore, the broad interaction between HSP27, HSP60, HSP70 and actin or tubulin under stress was very interesting. It is possible that the recently synthesized HSPs are recruited into the cytoskeleton. Such recruitment does not prevent collapsing of the filament network (Perng *et al*., 1999). HSPs recruitment facilitates microtubule and microfilament re-polymerization. This notion is supported by the findings of Jia *et al.* (2005) who used proteomic analysis of pseudopodial protrusions of MDCK cells, and described interactions between chaperones and actin or tubulin. Such interactions are mainly localized in the stress fibres. The cytoskeleton reorganization consists of an activation signal that induces actin polymerization and myosin phosphorylation followed by a tension-dependent assembly of actin and myosin into the stress fibres. Cross-bridging between actin and myosin filaments generates the tension (Deshpande *et al*., 2006) and it seems appear that HSPs participate in this process. Finally, it is concluded that stress increases HSP expression. HSPs are recruited into the stress fibres and are possibly involved in the actin-based filament re-polymerization.

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