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The HspA2 Protein Localizes in Nucleoli and Centrosomes of Heat Shocked Cancer Cells

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Abstract The human *HSPA2* gene, which belongs to the HSP70 family of heat shock genes, is a counterpart of rodent testis-specific *HspA2* gene. Rodent genes are expressed mainly in pachytene spermatocytes, while transcripts of human *HSPA2* gene have been detected in various normal somatic tissues, albeit translation of the messenger RNA into corresponding protein has not been yet unambiguously demonstrated, except for several cancer cell lines. The aim of our work, a first step in search for HspA2 function in cancer cells, was to establish its intracellular localization at physiological temperature and during heat shock. First, we used qRT-PCR and a highly specific antibody to select cell lines with the highest expression of the HspA2 protein, which turned out to be A549 and NCI-H1299 lines originating from non-small cell lung carcinoma (NSCLC). Significant expression of the HspA2 was also detected by immunohistochemistry in primary NSCLC specimens. Intracellular localization of the HspA2 was studied using both the specific anti-HspA2 polyclonal antibody and transfection of cells with fusion proteins HspA2-EGFP and mRFP-HspA2. We found that, at physiological temperature, the HspA2 was localized primarily in cytoplasm whereas, during heat shock, localization shifted to nucleus and nucleoli. Moreover, we demonstrate that in heat-shocked cells HspA2 accumulated in centrosomes. Our results suggest that the HspA2, like Hsp70 protein, can be involved in protecting nucleoli and centrosomes integrity in cancer cells subjected to heat shock and, possibly, other cellular stressors. J. Cell. Biochem. 104: 2193–2206, 2008.

Key words: HspA2; intracellular localization; heat shock; cancer cell lines; nucleus; nucleolus; centrosomes

The human *HSPA2* gene belongs to a HSP70 multigene family of heat shock genes [Bonnycastle et al., 1994] and is considered to be a counterpart of mouse and rat testis-specific *HspA2* genes [Krawczyk et al., 1988a,b; Zakeri et al., 1988; Ścieglińska et al., 1997, 2004]. Deletion of the *HspA2* in mice leads to massive apoptosis of spermatocytes and male infertility [Dix et al., 1997]. In the mouse, the product of the *HspA2* gene is a molecular chaperone essential for progression of meiosis, and is involved in synaptonemal complexes disassembly, formation of active CDC2/cyclin B1 complex in spermatocytes [Dix et al., 1996; Zhu et al., 1997], as well as in chaperoning transition proteins 1 and 2 in spermatids [Govin et al., 2006]. In a majority of extra-testicular tissues of adult rodents, the *HspA2* expression is either very low or fully repressed, and relatively high level of transcripts has been found only in the brain [Widłak et al., 1995; Murashov and Wolgemuth, 1996; Ścieglińska et al., 1997]. Recently, expression of the mouse *HspA2* gene during embryogenesis was also reported [Rupik et al., 2006].

In human testis the HspA2 was found in maturating spermatides and in sperm as well as, at low levels, in spermatocytes [Huszar et al., 2000]. Reduced expression of this protein was

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found to be related to male infertility [Huszar et al., 2000; Yesilli et al., 2005; Cedenho et al., 2006]. Contrary to rodents, relatively higher levels of *HSPA2* gene transcripts were reported to be present in various human somatic tissues [Bonnycastle et al., 1994]. Some level of the *HSPA2* gene activity was also observed in cell lines derived from several human cancers [Rohde et al., 2005; Piglowski et al., 2007], and in breast cancer surgical samples [Rohde et al., 2005]. However, the level of HspA2 protein expression in non-testicular cells is poorly characterized. Two antibodies were used to trace the HspA2 protein expression in somatic tissues: one produced against the NH2-SKLYQGGP-GGGGSSGGPT peptide (aa 611-628 of mouse HspA2 protein) [Son et al., 1999; Rohde et al., 2005] and the other directed against the CK-M/HspA2 protein isolated from sperm [Huszar et al., 2000]. Essentially, none or insignificant expression of the HspA2 was found in several normal human tissues [Son et al., 1999], while low expression was shown in HeLa and HBL-100 cell lines [Rohde et al., 2005].

So far, no studies concerning intracellular localization of the HspA2 protein were reported, although such knowledge could be important for speculating on the possible functions of this protein in cancer cells. Here, in order to determine where the HspA2 protein is present at physiological temperature and how does heat shock affect its localization, we used two experimental approaches: immunocytochemistry with a highly specific anti-HspA2 rabbit antiserum designed and purified by us, and transfection with plasmids encoding HspA2 tagged with fluorescent EGFP and mRFP proteins. For these experiments, we selected lung carcinoma cell lines A549 and NCI-H1299 which we found to express *HSPA2* gene at significant levels.

Our data indicate that, during heat shock, HspA2 protein translocates from cytoplasm into nuclei and nucleoli and that it also accumulates at centrosomes. These results suggest that HspA2 protein could be, similarly as Hsp70 protein, a part of chaperone system involved in protecting crucial cellular structures against damage induced by heat shock and possibly other stressors.

MATERIALS AND METHODS

Cell Lines

Human cancer cell lines: HCT116 (colon carcinoma), NCI-H1299 (non-small cell lung carcinoma), NCI-H358 (non-small cell lung carcinoma), NCI-H292 (lung mucoepidermoid carcinoma), A549 (lung carcinoma), MCF7 (breast epithelial adenocarcinoma), Me45 (melanoma); HepG2 (hepatocellular carcinoma) and human epithelial cell lines: HBL-100 (breast myoepithelial tumor) MCF-10A (immortalized human mammary epithelial cells) and BEAS-2B (virus transfected normal bronchial epithelium) were used. The sources of cell lines used are indicated in Acknowledgments Section. NCI-H1299, NCI-H358, NCI-H292, MCF7, A549 and BEAS-2B cell lines were grown in RPMI 1640 medium (Sigma-Aldrich, Inc.). HepG2, HBL-100, and MCF-10A cell lines were grown in DMEM (Sigma-Aldrich, Inc.) and HCT116 cells were grown in McCoy medium (Sigma-Aldrich, Inc.). All growth media were supplemented with 10% fetal bovine serum (ICN) and 40 μg/ml gentamycin. Growth medium for MCF-10A cells was additionally supplemented with 10 μg/ml insulin, 10 ng/ml EGF, and 100 ng/ml hydrocortisone, whereas that for BEAS-2B cells with 0.5 ng/ml EGF, 500 ng/ml hydrocortisone, 0.005 mg/ml insulin and for HBL-100 with 250 ng/ml insulin and 20 ng/ml EGF, respectively.

Heat shock was induced by adding prewarmed (42°C) growth medium to cells grown on Lab-Tek® Permanox® Chamber Slides (Nalgen Nunc International, Rochester, NY) followed by incubation of slides in a water bath at 42°C for the indicated time periods. Then the cells were fixed (as indicated below) and processed for immunochemistry.

Tissues

Paraffin blocks containing post-surgical specimens from our collection were used in order to determine immunohistochemically whether HspA2 is expressed in NSCLC. These NSCLC specimens were already characterized for expression of Hsc70, Hsp70, Hsp27, and various other proteins. Detailed description of these samples can be found in our earlier articles [Malusecka et al., 2001, 2006]. Post-surgical specimens of human testis (gift from Dr. R. Nowak) were used with Local Ethics Committee permission, as described earlier [Piglowski et al., 2007]. Samples of testis tissue were used as a source of antigen for purification of anti-HspA2 antiserum.

Real-Time Quantitative PCR

Total RNA was prepared using the guanidine isothiocyanate method [Chomczynski and Sacchi, 1987]. RNA samples were purified from contaminating DNA by on-column treatment with RNase-free DNase I (QIAGEN). After inactivation of the enzyme, a control PCR for DNA contamination was performed using GAPDH primers (GAPDH-F 5′CGTCTTCACCACCATGGAGA3′ and GAPDH-R 5′CGGCCATCACGCCACAGTTT3′). RNA concentration was measured using NanoDrop ND1000 (NanoDrop Technology). The integrity of RNA was analyzed using 2100 Bioanalyzer (Agilent); only RNA samples with the RNA Integration Number (RIN) higher than 8 were used for real-time Q-PCR. cDNA was synthesized from 500 ng of total RNA by Omniscript kit (QIAGEN). Fluorescent LNA probes (Universal Probe Library, Roche) were used with appropriate primers designed according to manufactures instructions in 5′-3′ direction. The *HSPA2* transcript was amplified using probe no. 70 (Universal Probe Library) and primers HSPA2-F, AAACTTTACCAAGGTGGTCCTG, HSPA2-R, GCTTAGTCCACTTCTTCGATGG. As reference genes, we amplified *GUS-B* (β-glucuronidase, probe no. 57 and primers GUSB-F, CGCCCTGCCTATCTGTATTC, GUSB-R, TCCCCACAGGGAGTGTGTAG), *B2M* (β-2 microglobulin, probe no. 42 and primers B2M-F, TTCTGGCCTGGAGGCTATC, B2M-R TCAGGAAATTTGACTTTCCATTC) and *ACTB* (β-actin, probe no. 11 and primers ACTB-F, ATTGGCAATGAGCGGTTC, ACTB-R, GGATGCCACAGGACTCCAT). PCR (20 μl volume) was carried out using commercial master mix formulation (Applied Biosystems) and ABI SDS 7700 machine, according to manufacturer's protocol. Reactions were performed in triplicate. Quantification was performed according to a standard curve obtained from serial dilutions of Human Reference RNA (Stratagene). Expression of the examined genes was normalized to the reference index, obtained by calculating geometric mean of reference genes' expression: *GUS-B*, *B2M* and *ACTB* (β-actin).

Recombinant Plasmids

For the purpose of this study we constructed expression vectors described below. The pEF1/HSPA2 plasmid used to overexpress wt-HspA2 in transfected cells was constructed by in-frame insertion of the *HSPA2* coding sequence between *Spe*I and *Sfu*I sites of the pEF1/V5-His B plasmid (Invitrogen). The *HSPA2* coding sequence was amplified by PCR using primers containing additional sequences for restriction enzymes suitable for subcloning. The pEF1/HSPA2-V5-His plasmid which encodes HspA2-V5-His fusion protein was constructed by in-frame insertion of the *HSPA2* coding sequence (with mutated stop codon) between *Spe*I and *Xba*I sites of pEF1/V5-His B plasmid. The pEF1/HSPA2-EGFP plasmid which encodes HspA2-EGFP fusion protein was constructed by inserting *EGFP* gene coding sequence (including *EGFP* stop codon) into the *Bst*BI restriction site of pEF1/HSPA-V5-His plasmid. The *EGFP* sequence was PCR-generated on the pEGFP-1 plasmid template (GenBank Accession no. U55761; Clontech Laboratories, Inc., Palo Alto, CA). The pEF1/mRFP-HSPA2 which encodes mRFP-HspA2 fusion protein was constructed by inserting *mRFP* coding sequence (with mutated stop codon) into the *Spe*I restriction sites of pEF1/HSPA2 plasmid. The *mRFP* coding sequence was generated on pRFP1-Control plasmid template (gift from Dr. M. Rusin). Transcription of all constructed genes was driven by the constitutive human *EF1α* (Elongation Factor 1-α) promoter. The correctness of the structure of all generated expression vectors was confirmed by sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystem). Details on plasmid construction are available on request.

Transfections

Cell lines constitutively overexpressing wt-HspA2 or HspA2 fusion proteins were established by transfecting NCI-H1299 cells with plasmids: pEF1/HSPA2 or pEF1/HSPA2-V5-His or pEF1/HSPA2-EGFP or pEF1/mRFP-HSPA2, respectively. All plasmids used in transfection experiments were purified using QIAfilter Plasmid Kit (QIAGEN) according to manufacturer's instruction. NCI-H1299 cells were seeded on 35 mm culture dish (Nalgen Nunc International), and transfected using Dharmafect (Dharmacon) reagent, according to manufacturer's protocol. Stably transfected cells were selected using culture medium supplemented with G418 (600 μg/ml) for 3 weeks. The expression of introduced genes and homogeneity of established cell lines were

confirmed by Western blot and immunocytochemistry.

Production and Purification of Rabbit Anti-HspA2 Serum

Serum against HspA2 protein was obtained by immunizing rabbits with NH2-SKLYQGGPGGGGSSGGPT peptide corresponding to amino acids 611–628 in the mouse HspA2 sequence (NCBI protein Acc No. NP_032327). Peptide was C-terminal-conjugated to bovine thyreoglobulin *via* the EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) method (Sigma-Genosys Custom Peptide Antisera Facility). The anti-HspA2 serum was purified by antigen affinity purification according to [Sambrook et al., 1989]. Briefly, frozen human testes or NCI-H1299 cells stably overexpressing wt-HspA2 protein (NCI-H1299(pEF1/HSPA2) cell line) were homogenized on ice in 3–5 volumes of buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 50 mM NaF, 1 mM DTT, 1 mM PMSF, a protease inhibitor mixture (Complete™, Roche) and centrifuged at 15,000*g* for 10 min at 4°C. Supernatant was aliquoted and total protein content was determined using Protein Assay Kit (BioRad). Protein (1.2 mg) extract from human testes was separated by SDS–PAGE on 8% polyacrylamide gel and immobilized on nitrocellulose membrane. Membrane fragment containing 70 kDa proteins was excised and incubated for 4 h at RT with rabbit HspA2-antiserum diluted in 5% milk in TBS (50 mM Tris (pH 7.0), 150 mM NaCl). After incubation with antiserum the membrane was washed three times for 5 min by TBS buffer supplemented with Tween-20 to final concentration of 0.025%. Next, antigen bound antibody was eluted by incubation in 200 mM glycine (pH 2.8) solution for 25 min and neutralized by addition of 1 M Tris (pH 7.5) to 100 mM final concentration. Anti-HspA2 antibody was aliquoted and stored at −20°C. Specificity of each stock of purified anti-HspA2 serum was confirmed by Western blot and immunocytochemistry (see Figs. 2 and 3).

Protein Extraction and Western Blotting

To prepare total protein extracts cells were grown in 60 mm cell culture dishes (Nalgen Nunc International) to 80% confluence. Then cells were harvested and lysed in 3–5 volumes of buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 50 mM NaF, 1 mM DTT, 1 mM PMSF and protease inhibitor mixture (Complete™, Roche). After incubation on ice (20 min) lysate was centrifuged at 15,000*g* for 10 min at 4°C. To isolate proteins from cytosol and nuclei, cells were washed in PBS, suspended in 200 μl of EC buffer containing 20 mM Tris (pH 7.6), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA, 2.5% glycerol, 0.5% NP-40, a protease inhibitor mixture (Complete™) and incubated on ice for 20 min. Next, samples were centrifuged at 330*g* at 4°C for 10 min. Supernatant containing cytosol proteins was transferred into new tube while pellet containing nuclear proteins was resuspended in 40 μl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 7.4)), incubated on ice for 20 min and centrifuged at 15,000*g* at 4°C for 5 min. Total protein content was determined using a Protein Assay Kit (BioRad) and 20–50 μg of proteins were fractionated by SDS–PAGE on 8% polyacrylamide gels and blotted onto nitrocellulose (BA85 Schleicher & Schuell, Dassel, Germany), blocked in 5% non-fat milk in TTBS (0.25 M Tris–HCl (pH 7.5), 0.1% Tween-20, 0.15 M NaCl) for 60 min and incubated overnight at 4°C with either anti-HspA2 antibody (this article), anti-Hsp70 antibody (Spa810, Stress Gene Biotechnology), anti β-actin antibody (Sigma-Aldrich, Inc.) or anti Werner's syndrome helicase WRN antibody (Novus Biologicals, Inc.). Primary antibodies were detected by goat anti-rabbit horse-radish peroxidase conjugated secondary antibody (Pierce) and visualized using SuperSignal® West Pico Chemiluminescent Substrate Kits (Pierce).

Immunocytochemistry, Immunohistochemistry and Monitoring of mRFP and EGFP Fluorescence

Cells grown on 4-well Lab-Tek® Permanox® Chamber Slides (Nalgen Nunc International) were fixed in methanol for 2 min at −20°C or in 4% formalin for 5 min at RT (where indicated), washed in PBS and incubated overnight with anti-HspA2 and/or with anti-γ-tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibodies. Anti-HspA2 primary antibody was detected using either goat anti-rabbit FITC conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) or ABC Vectastain kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine (Sigma-Aldrich,

Inc.) to visualize immunoreaction. Counterstaining was done with hematoxylin. Anti-γ-tubulin primary antibody was detected using goat anti-mouse rhodamine conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). To visualize genetic material, cells were incubated for 15 min in the darkness with 1 mg/ml DAPI (Sigma-Aldrich, Inc.) solution. The slides were washed twice in PBS and mounted in DAKO® Fluorescent Mounting Medium (DAKO). Cells were observed using an ECLIPSE E800 Nikon microscope. Pictures were recorded with a Hamamatsu CCD camera and an image analysis system (Lucia) and edited using Adobe Photoshop 6.0 program.

Post-surgical specimens of NSCLC [Malusecka et al., 2001, 2006] were fixed in 10% formalin and embedded in paraffin. To enhance imunohistochemical specificity before immunostaining, antigen retrieval was performed by two 5-min cycles in boiling 0.01 citrate buffer in a microwave oven [Malusecka et al., 2006]. The endogenous peroxidase activity was blocked with 1% H_2O_2 in PBS and slides were incubated overnight with monoclonal anti-Hsp70 SPA810 (StressGen Biotechnologies) according to Malusecka et al. [2006] and with purified anti-HspA2 primary antibodies. Biotinylated secondary antibody, an avidin–biotin complex (Vector Laboratories) and 3, 3′-diaminobenzidine (Sigma-Aldrich, Inc.) as chromogen were applied for visualization of immunoreaction. Identical procedure performed with omission of the primary antibody was considered as a negative control.

RESULTS

Expression of the HspA2 in Cancer Cells

In our previous work we showed that the transcription of the human *HSPA2* gene is initiated from a single start site localized 109 bp upstream of the ATG codon, and that the same *HSPA2* transcript is synthesized both in testicular cells and cancer cell lines [Piglowski et al., 2007]. Here, using quantitative real-time RT-PCR, we compared the amount of the *HSPA2* transcript in several human cancer cell lines. The data presented in Figure 1 show that the *HSPA2* transcript levels differ significantly between the cell lines tested. Significant expression of the gene was found in A549, NCI-H1299, HBL-100 and MCF-7 cell lines, while others can be considered

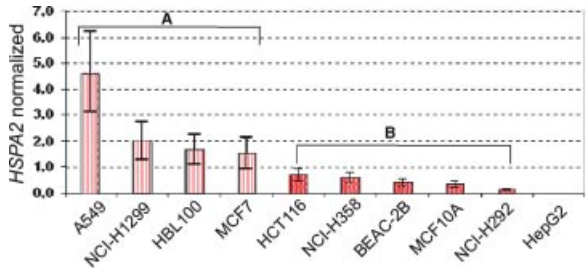

Fig. 1. Quantification of the *HSPA2* transcript levels in various human cell lines by real-time RT-PCR. Fluorescent LNA probes (Universal Probe Library, Roche) were used with appropriate primers (for details see ''Materials and Methods'' Section). Expression of the *HSPA2* gene was normalized to the reference index, obtained by calculation of geometric mean of reference genes expression: *GUS-B* (β-glucuronidase), *B2M* (β-2 microglobulin) and *ACTB* (β-actin). Error bars indicate standard deviation (n = 3). The difference between groups A and B was statistically significant, $P = 0.0167$ (calculated by Kruskal–Wallis, ANOVA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

as "low *HSPA2* expressors." The highest *HSPA2* transcript level was found in A549 cells, while the lowest (undetectable) in HepG2 cells.

To check whether the level of *HSPA2* transcription corresponds to the level of HSPA2 protein, we performed Western blot analysis (Fig. 2) using rabbit polyclonal antiserum raised against a peptide derived from the C-terminal amino acids 611–628 of mouse HspA2 and carefully purified by antigen affinity method (for details see Materials and Methods Section). Excellent specificity of this antibody was demonstrated by no cross-reactivity with Hsp70 protein (Fig. 2A) nor Hsc70 (not shown). Also, we have never observed (after heat shock) increased intensities of HspA2 on Western blots probed with our antibody, neither in cell lines expressing, nor in non-expressing the HspA2 protein (not shown). These results indicate that our anti-HspA2 antibody does not detect any inducible HSP70 family protein, including HspA6.

Western blotting, followed by densitometric analysis (Fig. 2B) of three independent immunoblots, confirmed particularly high expression of *HSPA2* gene in A549 lung cancer cells (Fig. 2). Significant levels of HspA2 protein were also present in NCI-H1299, NCI-H358 and MCF-7 cells. In the HBL-100, MCF-10A and BEAC-2B immortalized epithelial cell lines the HspA2 protein level was barely detectable. No HspA2 protein was detected in HepG2, NCI-H292 or HCT116 cells. In general, the level of the HspA2 protein corresponded to the

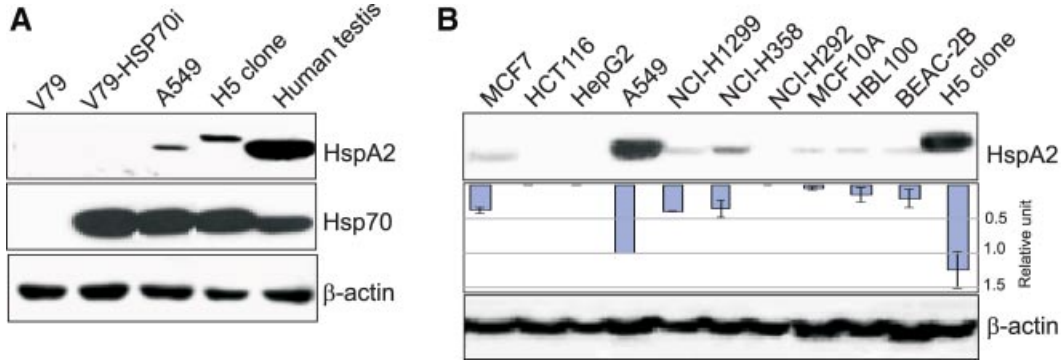

Fig. 2. The HspA2 protein expression level in various human cell lines. **A**: Verification of the specificity of rabbit polyclonal anti-HspA2 serum (antigen affinity purified, for details see ''Materials and Methods'' Section). Blots were incubated with antibodies: anti-HspA2, anti-Hsp70 (SPA810, StressGene Biotechnologies) or anti-β-actin (Sigma-Aldrich, Inc.). The latter one was used as a control of protein content uniformity. Cell lines used: V79-Hsp70i are V79 cells stably transfected with plasmid encoding Hsp70 protein [Glowala et al., 2002]; H5 are NCI-H1299 cells stably transfected with vector encoding HspA2-V5-His fusion protein. **B**: Western blot analysis of HspA2 expression in various human cell lines. Middle graph shows densitometric analysis of three independent immunoblots (Analysis program, SIS GmbH, Germany). Signals were normalized relative to the value obtained for A549 cells (annotated as 1) and are expressed in relative units. Error bars indicate standard deviation (n = 3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

transcription level with the exception of HCT116 cells. In this case PCR-based cloning of the transcription unit of the *HSPA2* gene from HCT116 cells and subsequent sequencing revealed no mutation in the coding sequence and in 5′ UTR region (data available on request). These results strongly suggest that in HCT116 cells the *HSPA2* gene expression is subjected to post-transcriptional control that hampers synthesis of the HspA2 protein.

Specificity of the antibody and reliability of our Western blot analysis was also confirmed by immunocytochemical detection of the HspA2 protein in selected cell lines defined as non-expressing (NCI-H292), low expressing (NCI-H1299) and highly expressing (A549) ones, as well as in NCI-H1299(pEF1/HSPA2) cells genetically modified to constitutively and strongly express wt-HspA2 protein under the control of human *EF1α* promoter (Fig. 3). It can be seen that immunocytochemistry did not reveal antigen presence in NCI-H292 cells in which neither expression of HspA2 could be detected by Western blot analysis. Weak HspA2 immunostaining was observed in the cytoplasm and nucleus of wild-type NCI-H1299 cells. In contrast, a significant immunostaining could be seen in A549 cells, in accordance with the data obtained from RT-PCR and Western blot analysis. As expected, a very strong immunostaining was present within NCI-H1299(pEF1/HSPA2) cells. These results clearly show that the anti-HspA2 antibody recognizes specifically and reliably the HspA2 not only on Western blots but also in immunocytochemical preparations.

The observation that cell lines derived from lung tumors express high levels of HspA2 prompted us to test whether this protein is also detectable in primary lung cancer. For immunohistochemical analysis of HspA2 we chose several samples of paraffin-embedded surgical specimens of NSCLC which in our earlier analysis were shown not to express

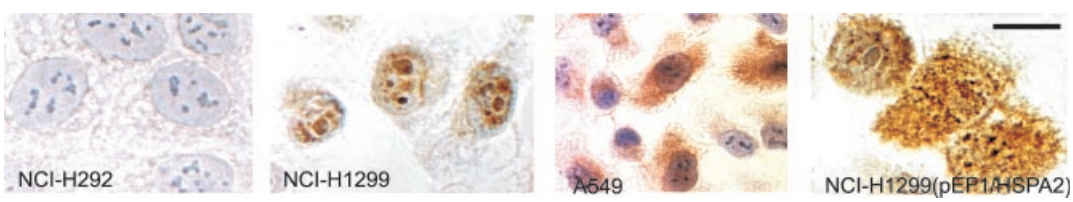

Fig. 3. Immunocytochemical detection of the HspA2 protein in selected cell lines with purified anti-HspA2 serum. Cells were methanol-fixed, the immunoreaction was performed by ABC method as described in ''Materials and Methods'' Section. Cells were counterstained with hematoxylin. Bar, 20 μm.

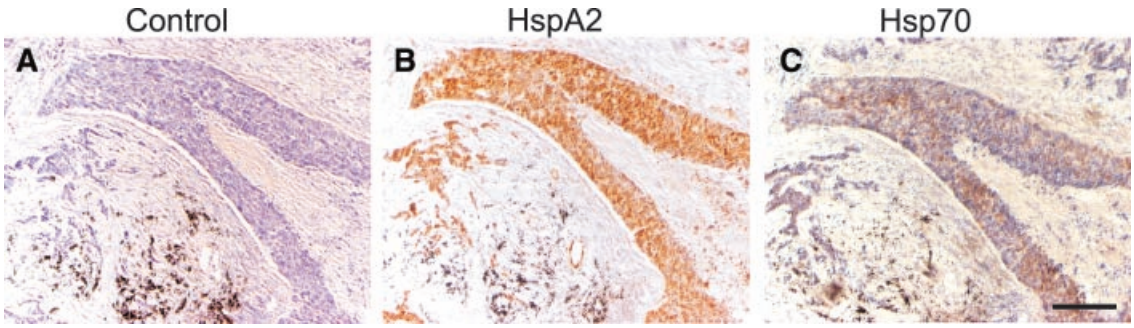

Fig. 4. Immunohistochemical detection of the HspA2 protein in primary non-small cell lung carcinoma. The paraffin sections of squamous cell carcinoma were stained by ABC method as described in ''Materials and Methods.'' **A**: Control reaction without primary antibody staining. **B**: Anti-HspA2 staining. **C**: Anti-Hsp70 staining (SPA810 antibody, StressGene Biotechnologies). All sections were counterstained with hematoxylin. Bar, 200 μm.

inducible Hsp70 protein [Malusecka et al., 2001, 2006]. We found that the HspA2 protein is highly expressed in some primary NSCLC (example of HspA2-positive sample is shown in Fig. 4). Extensive analysis of the HspA2 expression pattern in NSCLC and its possible correlation with clinical features was not the aim of this study and will be presented elsewhere (Ścieglińska et al., in preparation).

Intracellular Localization of the HspA2 Protein

As a first step in search for HspA2 protein function in somatic/cancer cells we decided to determine intracellular localization of the protein using A549 cell line (in which the intrinsic level of the *HSPA2* gene was the highest of all cell lines tested), at physiological temperature and under heat shock conditions. First, we analyzed by Western blotting the levels of HspA2 protein in cytoplasmic *versus* nuclear fractions at various time points following heat shock. We found that at physiological temperature the HspA2 protein was localized primarily in the cytoplasm, whereas during heat shock the level of nuclear HspA2 gradually increased (Fig. 5A). It has to be noted that the expression of the *HSPA2* gene is not induced by elevated temperature (our unpublished results; see also Tang et al. [2005]), thus increased HspA2 levels in the nuclear fraction result from its translocation from cytoplasm into nucleus.

Subsequent immunocytochemical analysis performed with anti-HspA2 antibody revealed that, in heat-shocked cells, the immunostain was accumulated within the nuclei and concentrated in subnuclear structures, presumably nucleoli. Interestingly, we also observed in heat-shocked cells a clear HspA2 immunostaining in structures that could correspond to centrosomes of mitotic cells (Fig. 5D). It has to be noted that although the pattern of the intracellular immunostain distribution was similar, the intensity of the immunostaining of cells was variable. It is commonly known that the A459 cell line, derived from explant culture of human lung adenocarcinoma [Giard et al., 1973], is phenotypically heterogeneous and contains various subpopulations that differ in morphology, growth behavior, gene expression profiles, tumorigenicity and sensitivity to toxins [Croce et al., 1999; Watanabe et al., 2002]. We believe that this feature of A549 cells can also explain variability in the HspA2 expression level revealed by immunostaining.

In order to confirm nucleolar and centrosomal localization of the HspA2 we constructed expression vectors pEF1/HSPA2-EGFP and pEF1/mRFP-HSPA2 encoding HspA2-EGFP and mRFP-HspA2 fusion proteins (Fig. 6A). These vectors were used for stable transfection of NCI-H1299 cells. We used NCI-H1299 cell line due to homogeneity of HspA2 expression, excellent transfection efficiency and rapid selection of transfectants.

Stably transfected cell lines, called by us NCI-H1299(pEF1/HSPA2-EGFP) and NCI-H1299 (pEF1/mRFP-HSPA2), expressed constitutively high levels of fusion proteins HspA2-EGFP and mRFP-HspA2, respectively. Direct fluorescence of reporter proteins, mRFP and EGFP, was observed at physiological temperature over entire cell, with evidently increased intensity in the cytoplasm (Fig. 6B,F,J). On the contrary, when cells were subjected to heat shock, we observed a

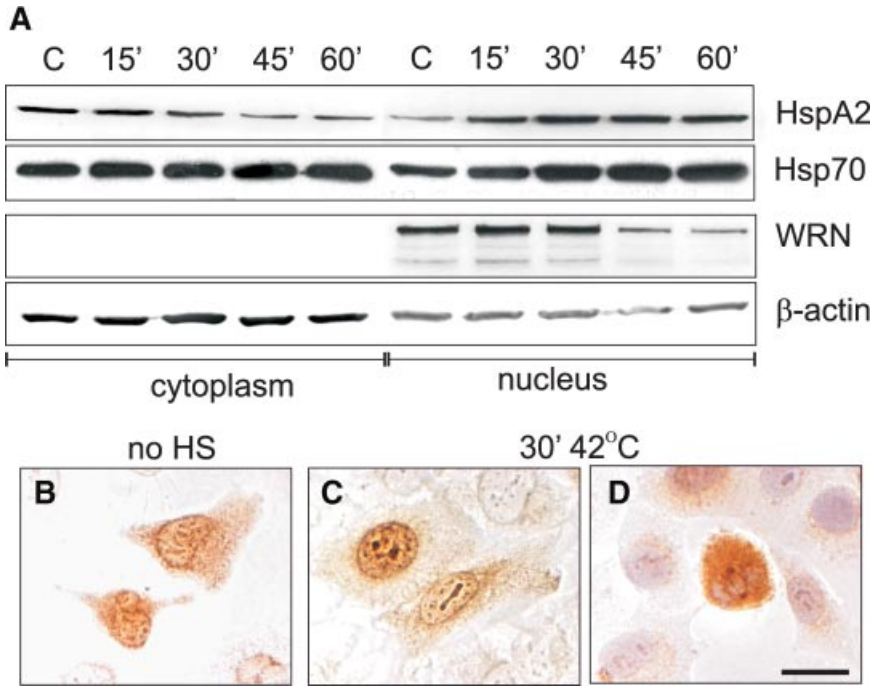

Fig. 5. Intracellular localization of HspA2 protein in A549 cells. **A**: Detection of HspA2 in cytoplasmic and nuclear fractions of A549 cells subjected to heat shock. Cells were heat shocked for 15 to 60 min. Cytosolic and nuclear fractions were isolated as described in the ''Materials and Methods'' Section and subjected to Western blotting with primary antibody against HspA2 or Hsp70 protein. WRN and β-actin were used as loading control. **B**–**D**: Immunocytochemical detection of HspA2 protein at physiological temperature (B) and after heat shock (C,D). Cells were stained with anti-HspA2 antibody using ABC method (B–D). In (D) cells were counterstained with hematoxylin. Phase contrast was used to better visualize cells morphology. Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

very effective translocation of mRFP-HspA2 proteins into the nucleus and subnuclear structures resembling nucleoli (Fig. 6H). By indirect fluorescence (using anti-HspA2 primary and FITC-conjugated secondary antibodies) we observed the same pattern of intracellular localization for wt-HspA2 protein when overexpressed in NCI-H1299(pEF1/HSPA2) cells (Fig. 6B,D).

Surprisingly, in contrast to mRFP-HspA2, the HspA2-EGFP fusion protein did not efficiently accumulate in nuclear structures of heat-shocked cells but remained evenly diffused throughout entire cell (Fig. 6J). Several studies were reported using Hsp70 and Hsc70 proteins tagged with EGFP on either C- or N-terminus [e.g., Dastoor and Dreyer, 2000; Zeng et al., 2004; Kodiha et al., 2005; Wang et al., 2008]. No significant differences were noted between the efficiency of nuclear relocation of any of these fusion proteins, as compared to wild-type ones. The reason for inefficient relocation of the HspA2-EGFP fusion protein is at present unknown.

To unequivocally identify subnuclear structures accumulating Hspa2 we coexpressed mRFP-HspA and B23 protein, a specific nucleolar marker. Figure 7 shows perfect colocalization of both proteins in heat-shocked cells, which proves that, after heat shock, the HspA2 is targeted to the nucleoli (Fig. 7).

As already shown above, observation of immunostaining of HspA2 in heat-shocked wild-type A549 cells suggested that this protein can also migrate into centrosomes (Fig. 5D). To verify whether HspA2 protein accumulates at centrosomes during heat shock we observed indirect fluorescence of wt-HspA2 and direct fluorescence of mRFP-HspA2 and HspA2-EGFP fusion proteins in NCI-H1299 cell lines overexpressing the indicated proteins. Accumulation of HspA2 in the centrosomal area was clearly visible during mitosis both in NCI-H1299(EF1/HSPA2) cells overexpressing wt-HspA2 (Fig. 8N,P) and in NCI-H1299 cell lines overexpressing HspA2-EGFP or mRFP-HspA2 fusion proteins (not shown). This reaction was very rapid as HspA2 concentration at centrosomes was visible within 10 min after mild (42°C) heat shock (Fig. 8F,H). The colocalization studies using γ-tubulin as a centrosomal

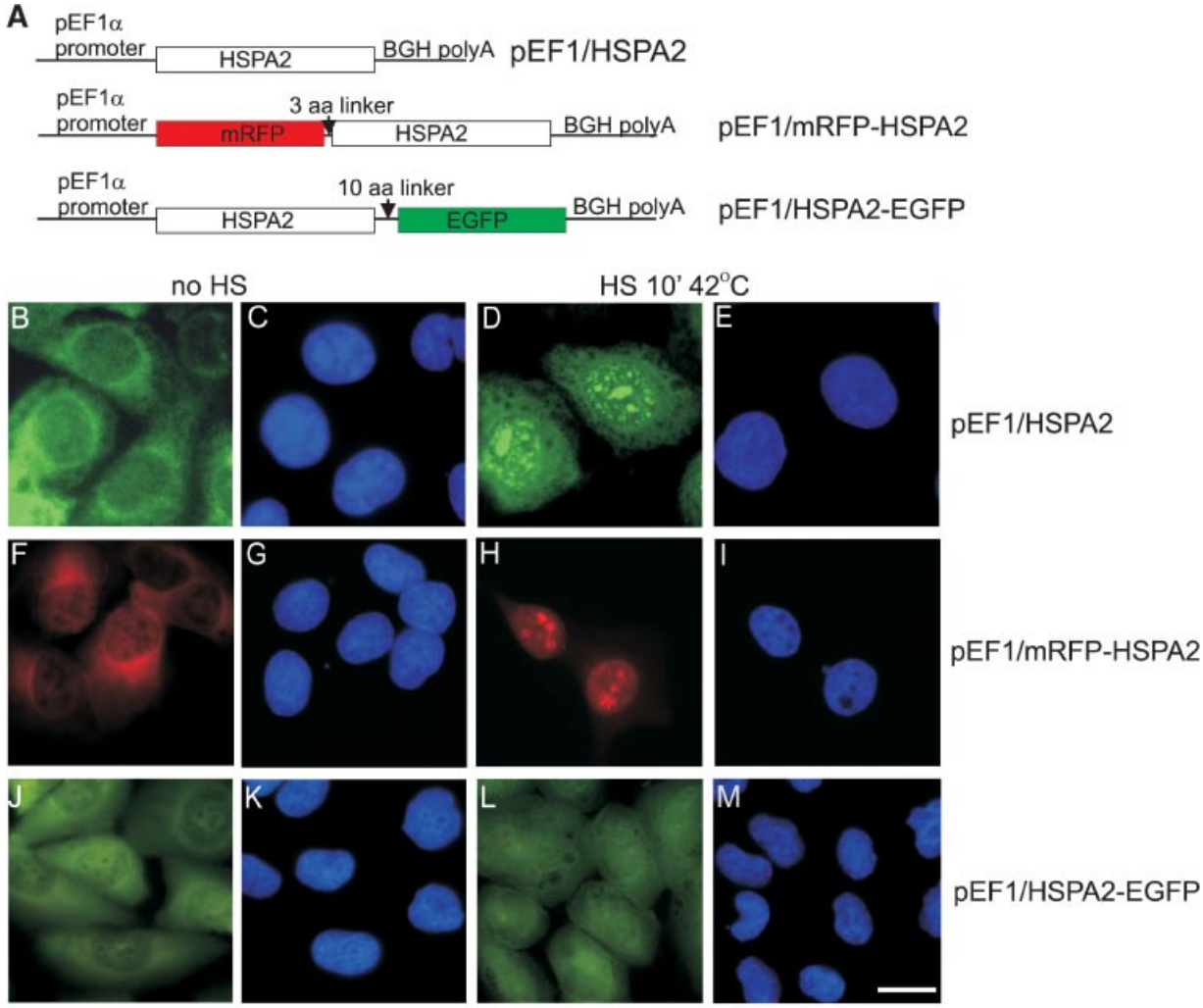

Fig. 6. Translocation of the HspA2 into nucleus and nucleolus. **A**: Construct legend: pEF1/HSPA2—plasmid encoding wt-HspA2 protein; pEF1/mRFP-HSPA2—plasmid encoding mRFP-HspA2 fusion protein; pEF1/HSPA2-EGFP—plasmid encoding HspA2-EGFP fusion protein (for details of plasmid construction see ''Materials and Methods'' Section). **B**–**E**: NCI-H1299 cells stably transfected with plasmid encoding wt-HspA2. Cells were methanol-fixed and stained with anti-HspA2 primary and FITC-conjugated secondary antibody. In (D) nuclear and nucleolar localization of the wt-HspA2 protein is visible in heat-shocked cells. **F**–**I**: NCI-H1299 cells stably transfected with construct encoding mRFP-HspA2 fusion protein. Cells were formalin-fixed and direct fluorescence of mRFP-HspA2 fusion protein was examined. In (H) nuclear and nucleolar localization of the mRFP-HspA2 is visible in heat-shocked cells. **J**–**M**: NCI-H1299 cells stably transfected with construct encoding HspA2-EGFP fusion protein. Cells were methanol-fixed and direct green fluorescence of HspA2-EGFP was observed. Note that translocation of HspA2-EGFP fusion protein into the nuclei is severely hampered in heat-shocked cells (L). Nuclei were DAPI stained (C,E,G,I,K,M). Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

marker clearly showed the area of HspA2 accumulation corresponds to centrosomes. These data did not exclude the possibility that the observed centrosomal accumulation of recombinant wt-HspA2 could be caused by its strong overexpression in transfected NCI-H1199 cells. To elucidate this ambiguity we immunostained endogenous HspA2 and γ-tubulin in A549 cells and we observed clear colocalization of both proteins at mitotic centrosomes after heat shock (42°C; Fig. 8N,P). However, in A549 cells the HspA2 accumulation at centrosomes was slower than in NCI-H1299(EF1/HSPA2) (30 min vs. 10 min of heat shock) presumably due to lower concentration of HspA2 in A549 cells.

Due to a spotty pattern of the HspA2 distribution within the nucleolar area in the cell lines expressing HspA2 or mRFP-HspA2, we could not clearly elucidate that HspA2 accumulates also at centrosomes of interphase cells. To unambiguously determine that HspA2 localizes

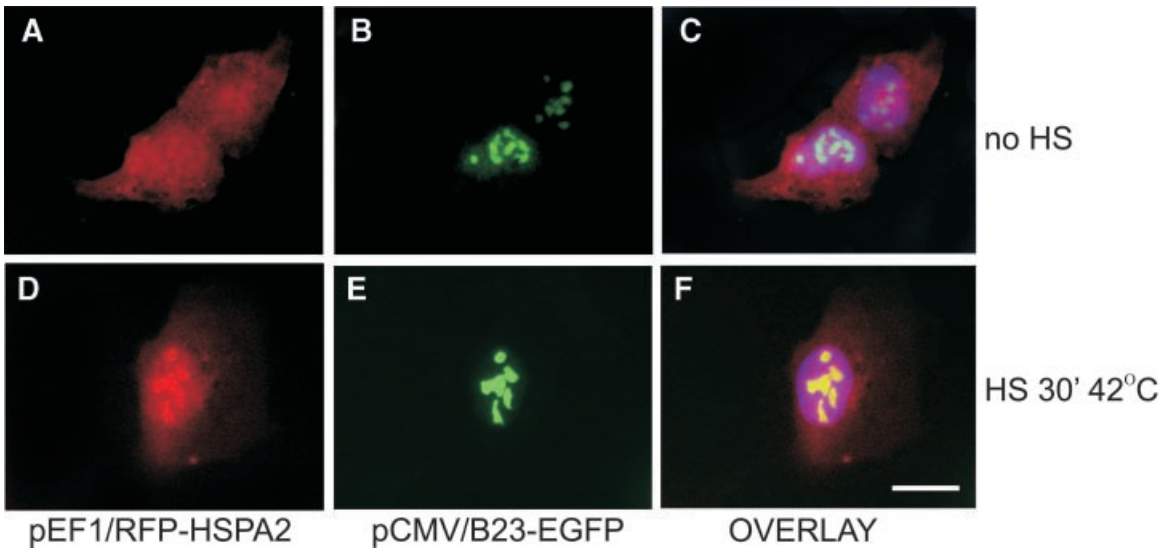

Fig. 7. Colocalization of HspA2 and nucleolar marker protein B23 in heat-shocked cells. NCI-H1299(pEF1/mRFP-HSPA2) cells constitutively expressing mRFP-HspA2 fusion protein were transfected with plasmid pCMV/B3-EGFP encoding B23-EGFP fusion protein. Cells were formalin-fixed and direct fluorescence of fusion proteins was examined. **A**–**C**: Cells were cultured at physiological temperature or (**D**–**F**) subjected to heat shock (30 min, 42°C). A,D: Red fluorescence of mRFP-HspA2 fusion protein. B,E: Green fluorescence of B3-EGFP fusion protein. C: Overlay of pictures A and B. F: Overlay of pictures D and E. Colocalization of B23-EGFP and mRFP-HspA2 fusion proteins is seen. Bar, 20 μm.

at centrosomes of interphase cells we decided to use NCI-H1299(pEF1/HSPA2-EGFP) cells due to inability of HspA2-EGFP fusion protein to enter nucleus during heat shock. In fact, after heat shock we observed either one or two bright-green spots localized very close to the nucleus (Fig. 9). The colocalization studies using γ-tubulin proved that these HspA2-EGFP spots correspond to interphase centrosomes. Kinetics of the HspA2-EGFP accumulation at centrosomes of interphase cells were exactly the same as for mitotic cells expressing wt-HspA2 or HspA2 fusion proteins. The centrosomal spots were hardly visible in stressed cells expressing wt-HspA2 or mRFP-HspA2, as their signal was obscured by strong fluorescence of HspA2 accumulating in nucleoli (data not shown).

DISCUSSION

Our study shows that, under heat shock conditions, HspA2 protein accumulates in the nuclei, nucleoli and centrosomes. The data suggest that HspA2 protein shares some biological properties with two members of the HSP70 family, namely Hsp70 protein coded by inducible *HSPA1A* and *HSPA1B* genes and Hsc70 protein coded by constitutive *HSPA8* gene. All these three Hsps are highly homologous and HspA2 shows 83.8% and 86.7% amino acids identity to Hsp70 and Hsc70, respectively [Bonnycastle et al., 1994]. Recent data from the genetic analysis indicated that the intronless *HSPA2* gene is a retroposed copy of the *HSPA8*/*HSC70* parental gene [Vinckenbosch et al., 2006].

At physiological temperature Hsc70 is constitutively expressed and localized mainly in cytoplasm. Similar intracellular distribution is shown by Hsp70 (if expressed), Hsc70 [Ellis et al., 2000], and HspA2 [this article]. The situation is different for NSCLC, the only primary tumor for which a comparison of intracellular distribution pattern of Hsc70 [Malusecka et al., 2001], Hsp70 [Malusecka et al., 2008] and HspA2 [Ścieglińska et al., in preparation] is available. In these cells all three Hsp70 proteins show clear preference for nuclear localization at physiological temperature. Thus, it seems justified to classify the HspA2, similarly to Hsp70 and Hsc70, as cytoplasmic/nuclear protein.

It has been well known that under heat shock conditions both Hsc70 and Hsp70 migrate into cell nucleus, with tendency to accumulate in nucleoli. It is thought that, under stress conditions, concentration of Hsp70 in the nucleus is a part of cellular protective response. The mechanism of HspA2 translocation into the

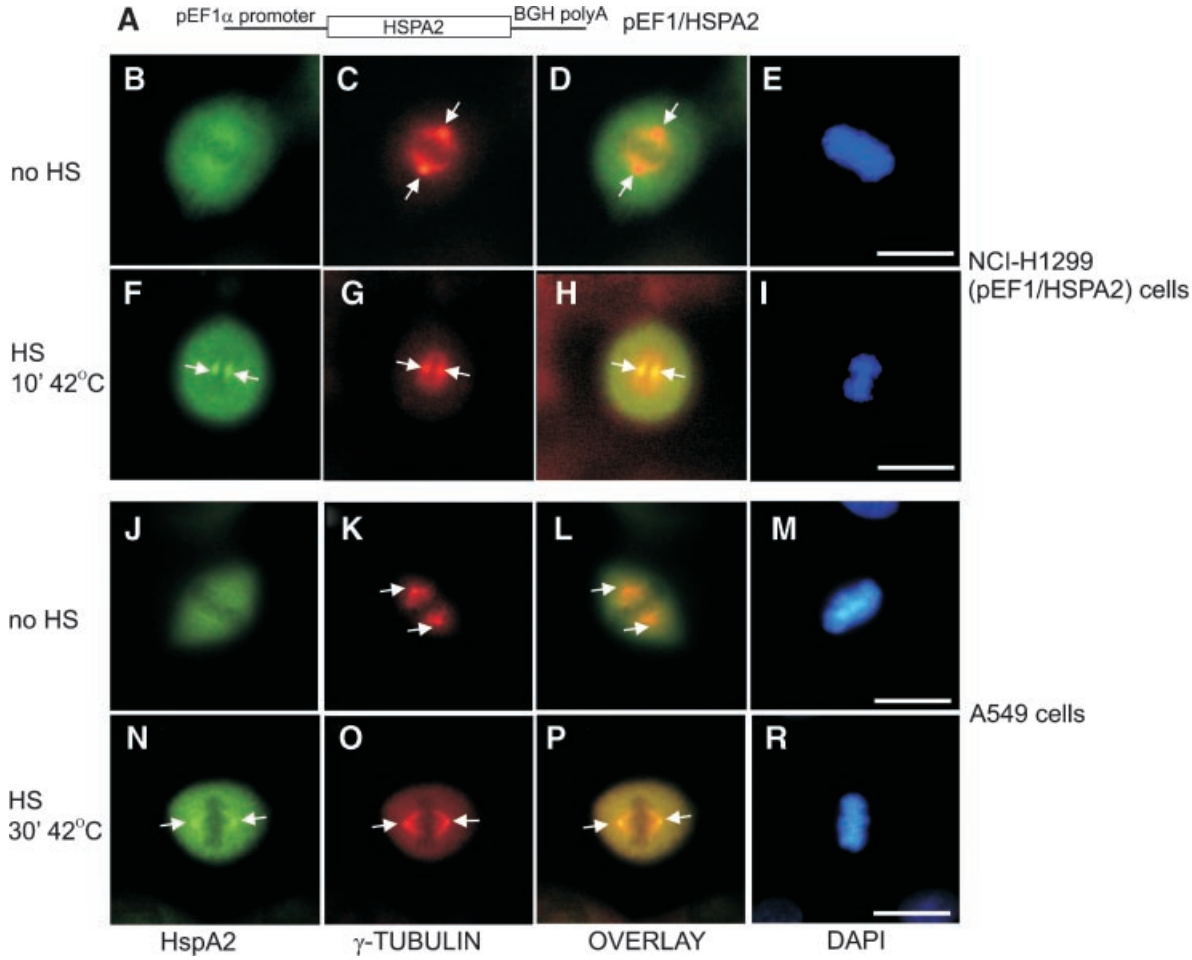

Fig. 8. Accumulation of HspA2 at centrosomes of mitotic cells during heat shock. **A**: Scheme of pEF1/HSPA2 gene construct coding for wt-HspA2 (for details of plasmid construction see ''Materials and Methods'' Section). **B–I**: wt-HspA2 protein constitutively expressed in NCI-H1299(pEF1/HSPA2) cells accumulates at centrosomes after heat shock (10 min, 42°C). **J–R**: Accumulation of endogenous HspA2 protein in centrosomes of A549 cells after heat shock (30 min, 42°C). Colocalization of wt-HspA2 (H) and endogenous HspA2 (P) and γ-tubulin is visible at centrosomes of cells subjected to heat shock. Cells were cultured at physiological temperature (B–E,J–M) or were subjected to heat shock (F–I,N–R). Cells were methanol-fixed and were probed with anti-HspA2 primary and FITC-conjugated secondary antibodies (B,F,J,N) and with anti-γ-tubulin primary and rhodamine-conjugated secondary antibodies (C,G,K,O). DNA was stained by DAPI (E,I,M,R). White arrows point at centrosomes. Bar, 20 μm.

nuclei/nucleoli, is presently unknown and can be only a matter of speculation.

Several functional studies found alleged NLS at different locations in the Hsp70/Hsc70 molecule showing divergent amino acid sequences [Lamian et al., 1996; Knowlton, 1999, 2001; Tsukahara and Maru, 2004] thus leading to inconsistent results. Existence of several so-called universal nucleolar retention signals (NoDS) has been suggested recently in various proteins including Hsc70 [Mekhail et al., 2000]. While multiple alleged NoDS are also present in HspA2 in similar locations as in Hsc70, it seems possible that these amino acid motifs used as relocation signals could be identical or similar for both proteins. However, a functional significance of theses putative NoDS has to be determined.

Considering intracellular localization of Hsc70, Hsp70, and HspA2, it can be speculated that various stimuli engage different translocation mechanisms. It has been shown that oxidative stress, which induces accumulation of Hsc70 within the nucleus, does not significantly affect intracellular distribution of Hsp70 [Dastoor and Dreyer, 2000]. It has been also observed that during early S phase of the cell cycle Hsc70, but not Hsp70, migrates into the

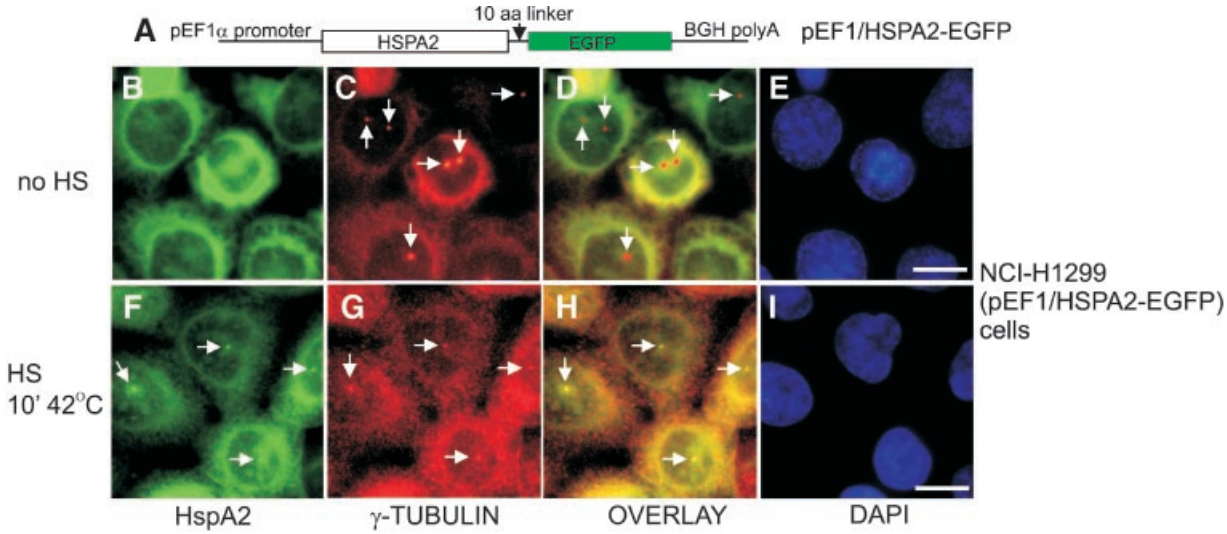

Fig. 9. Accumulation of HspA2 at centrosomes of interphase cells during heat shock. **A**: Scheme of pEF1/HSPA2-EGFP gene construct encoding HspA2-EGFP fusion protein (for details of plasmid construction see ''Materials and Methods'' Section). **B–E**: NCI-H1299(pEF1/HSPA2-EGFP) cells constitutively expressing HSPA2-EGFP protein were cultured at physiological temperature or (**F–I**) were subjected to mild heat shock (10 min, 42°C). B,F: Direct fluorescence of HspA2-EGFP. H: Colocalization of HSPA2-EGFP and γ-tubulin at centrosomes of interphase cells subjected to heat shock. Cells were methanol-fixed and were probed with anti-γ-tubulin primary and rhodamine-conjugated secondary antibodies (C,G). DNA was stained by DAPI (E,I). White arrows point at centrosomes. Bar, 20 μm.

nucleus [Zeise et al., 1998]. Existence of distinct or multiple, partially overlapping mechanisms of nuclear translocation for all three HSPs seems to be supported by observation that tagging of the C-end of the Hsc70 with GFP protein did not affect the rate of migration into the nucleus [Dastoor and Dreyer, 2000], whereas HspA2 tagged at C-terminus with GFP was retained in the cytoplasm (this article).

An intriguing observation is that of HspA2 shift into the centrosomes of heat-shocked cells. A search for the involvement of Hsp70 family proteins in the protection of centrosomes and mitotic spindle against heat-induced damages has relatively long history but only recently convincing results have been published [Hut et al., 2005]. The latter authors unambiguously demonstrated that heat shock induced transient accumulation of Hsp70 (both wild-type and plasmid-encoded) at mitotic centrosomes of OT70 hamster lung fibroblasts. Moreover, Hsp70 protected these cells against the majority of heat-induced division abnormalities. It seems that mainly stress-inducible Hsp70 was responsible for the observed effects; as for Hsc70, centrosomal localization was observed neither in the presence nor in the absence of heat shock stressor [Hut et al., 2005]. Whether accumulation of the HspA2 in centrosomes has any functional significance has to be determined. Such study is underway in our laboratory.

It is becoming evident that highly similar Hsp70 and Hsc70 proteins exhibit some significant functional differences. Besides the ones discussed above differences may also concern the aggregation status [Angelidis et al., 1999] and immunogenic properties as seen from differential affinity to antigenic peptides [Callahan et al., 2002]. It is thus possible that HspA2 may functionally overlap with Hsp70 and Hsc70 and, additionally, have its own specific functions. Recent findings indicate that Hsp70-depleted HeLa cells were arrested in G/M phase, whereas those depleted of HspA2 were arrested in G1 phase [Rohde et al., 2005]. Moreover, HeLa cells depleted either of HspA2 or Hsp70 revealed significant differences in the global gene expression profile [Rohde et al., 2005]. However, more data are needed in order to validate and generalize this observation.

The data provided herein form basis for further studies determining the function and action mechanism of HspA2 in cancer cells. An important problem to solve is how to determine functional interplay between HspA2, Hsp70 and Hsc70 proteins.

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