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Heat Shock Affects Trafficking of DAX-1 by Inducing Its Rapid and Reversible Cytoplasmic Localization

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DAX-1 is an unusual orphan nuclear receptor whose mutations cause adrenal hypoplasia congenita (AHC) associated with hypogonadotropic hypogonadism (HHG). Subcellular localization of DAX-1 is a critical determinant of its biological activity. Indeed, the missense mutants found in AHC patients have an impaired transcriptional repressor activity due to protein misfolding and shift of their localization to the cytoplasm. For this reason, we sought to identify factors that regulate DAX-1 subcellular localization. Of several stimuli and chemical compounds tested, heat shock was the only stimulus able to induce rapid and massive relocalization of DAX-1 in the cytoplasm. The heat shock effect is reversible and does not involve stimulation of the p38 and ERK pathways. Heat shock probably acts by inducing modifications of DAX-1 and increasing its partitioning in the insoluble cellular fraction.

Key Words: Heat shock; signaling; nuclear receptors.

Introduction

DAX-1 (NR0B1) *(1)* is an unusual member of the nuclear hormone receptor superfamily whose mutations cause the X-linked form of adrenal hypoplasia congenita (AHC), which is usually associated with hypogonadotropic hypogonadism (HHG) *(2,3)*. DAX-1 expression is restricted to steroidogenic tissues and to some critical sites in the reproductive axis *(4)*. The human *DAX-1* gene encodes a 470-amino-acid protein whose carboxyl (C) terminus is similar to the ligand-binding domain (LBD) of nuclear hormone receptors, while its amino (N) terminus is composed of three repeats of 67–69 residues with no significant similarity to any other known protein. DAX-1 is a transcriptional repressor of several genes involved in steroid hormone metabolism through a potent transcriptional repression domain present in its C terminus *(5–8)*. All *DAX-1* point mutations identified in patients with AHC alter the protein C terminus and invariably impair its transcriptional repression activity *(6,9)*. Recently, we have also shown that the DAX-1 AHC missense mutants are mislocalized in the cytoplasm, probably as a consequence of their misfolded conformation *(10, 11)*. DAX-1 localization changes during pituitary development from nucleocytoplasmic at embryonic day (E) 10.5 to nuclear at E 18.5 *(12)*.

Because DAX-1 subcellular localization is a critical determinant of its biological function, it is essential to understand the basis of its regulation. For this reason, we have investigated the implication of different signal transduction pathways and stimulations upon DAX-1 subcellular distribution. We observed that heat shock is the only stimulus affecting DAX-1 localization, working by shifting completely the localization of the DAX-1 protein toward the cytoplasm. DAX-1 heat shock–induced cytoplasmic localization requires the cooperation of the DAX-1 N- and C-terminal domains and is reversible. This effect is also independent of Crm1, p38, and ERK. We show here that heat shock acts by inducing modifications of DAX-1 and increasing its insolubility.

Results

Heat Shock Selectively Affects DAX-1 Subcellular Localization

The observation that the DAX-1 protein is shuttling between the nucleus and the cytoplasm and that its subcellular localization has three distinct patterns (nuclear, cytoplasmic, and nucleocytoplasmic) in transfected cells *(10)* suggests that DAX-1 subcellular localization may be regulated by signaling pathways or cell cycle progression. To study how different treatments control DAX-1 localization, we expressed DAX-1 in HeLa cells and treated the cells with various stimuli (Fig. 1A). Prior to stimulations, the cells were pretreated with an inhibitor of protein synthesis, cycloheximide (15 μg/mL starting from 30 min before and during

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all the time of stimulation), to make sure that the observed results are truly due to alterations in the kinetics of cycling between the compartments and not to a cytoplasmic accumulation of newly synthesized DAX-1. Cycloheximide treatment alone does not modify DAX-1 subcellular localization. As we have shown previously *(10,11)*, in untreated cells DAX-1 is predominantly nuclear, but its localization is cytoplasmic or nucleocytoplasmic in a subset of cells (about 20%). The distribution of DAX-1 is not modified by treatment with anisomycin (50 ng/mL for 30 min), sorbitol (0.6 *M* for 30 min), UV (500 mJ associated or not with a 30 min recovery period), arsenite at a sublethal (0.5 m*M*) or a lethal concentration (2 m*M* for 1 h), and H_2O_2 (200 μ*M* for 30 min and 1 h). Conversely, heat shock (10 min at 45°C) completely reverses the subcellular distribution of DAX-1, which becomes mainly cytoplasmic after only 10 min of shock.

The cells were also treated with chemical inhibitors or activators of diverse pathways (Fig. 1B): an inhibitor of the RNA polymerase actinomycin D (5 ng/mL for 3 h), the PI3 kinase inhibitor wortmannin (10 n*M* for 3 h), the PKA activator dibutyryl-cAMP (1 m*M* for 3 h), the ERK inhibitor PD 98059 (20 μ*M* for 1 h), and the p38 inhibitor SB 203580 (20 μ*M* for 1 h). None of these inhibitors or activators have an effect upon DAX-1 subcellular localization.

We also studied whether DAX-1 subcellular localization is dependent on cell cycle progression. In order to test this hypothesis, we checked DAX-1 localization immediately after 48 h of serum starvation or after serum starvation followed by the addition of serum (2.5%) or EGF (30 ng/mL) for 30 min or 1 h (Fig. 1C). No change in DAX-1 subcellular localization is observed in all these conditions. We can conclude that DAX-1 subcellular localization is not cell cycle-dependent.

Endogenous DAX-1 Is Also Responsive to Heat Shock

Considering the striking effect of heat shock on DAX-1 subcellular localization when the protein is transiently expressed in HeLa cells (Fig. 2A), we wanted to corroborate if the endogenous protein also shows the same behavior. Human adrenocortical H295R cells were used to study the endogenous DAX-1. As previously shown, DAX-1 is localized mainly in the nucleus but also in the cytoplasm in this cell line *(14)*. No staining is observed when using nonspecific IgG as primary antibody (data not shown). Incubation of H295R cells at 45°C for 10 min causes a reversion of DAX-1 subcellular distribution (Fig. 2B). Indeed, DAX-1 is localized mainly in the cytoplasm with an intense staining in the perinuclear region and cytoplasmic aggregates are visible in the form of small dots. As a control, we also checked the localization of the transcription factor CREB. Its nuclear localization is not changed by heat shock (data not shown).

The magnitude of the heat shock effect upon DAX-1 protein localization is cell-type-dependent: in transfected COS cells, the percentage of cells with cytoplasmic localization of DAX-1 after heat shock is higher than in HeLa cells (Fig. 2C). In COS cells we observed a heat shock effect that is proportional to the temperature (42°C or 45°C) and the duration (5 or 10 min) of the heat shock.

Both Isolated DAX-1 N- and C-Terminal Domains Display Cytoplasmic Localization Subsequently to Heat Shock

We next wanted to identify the role of the different domains of DAX-1 in the heat shock–induced redistribution of DAX-1. To this purpose, we studied the distribution of the truncated proteins corresponding to the N-terminal (1–205) and the C-terminal (205–470) domains of the DAX-1 protein transiently expressed in HeLa cells, in basal conditions or after heat shock treatment. For both the DAX-1 N-terminal and Cterminal domains, we observe at the basal state 5–10% of cells with a cytoplasmic localization (Fig. 3A). After heat shock, this percentage increases up to 50%, while the full-length protein is localized in the cytoplasm of 80% of the cells. These data show that both isolated DAX-1 N- and C-terminal domains are sensitive to heat shock–induced cytoplasmic redistribution and their effect is additive on the behavior of the full-length protein.

DAX-1 Heat Shock–Induced Nuclear Exclusion Is Independent of Crm1, p38, and ERK

The cytoplasmic localization of DAX-1 observed after heat shock could be due to a blockage of the nuclear import or an increase in the nuclear export. To discriminate between these two hypotheses, we treated the cells with the Crm1 inhibitor leptomycin B (20 ng/mL for 8 h). In transfected HeLa cells, the localization of full-length DAX-1 is not modified by leptomycin B in cells cultured in basal conditions and after heat shock (Fig. 3B). The same results were obtained with the DAX-1 N- and C-terminal domains (data not shown). We can conclude that the cytoplasmic localization observed in response to heat shock is not mediated by a Crm1-dependent export.

In addition, heat shock produces activation of the p38 kinase and extracellular signal-regulated kinases (ERKs) *(15,16)*. Therefore, we decided to investigate whether these pathways are implicated in the heat-induced relocalization. We exposed transiently transfected HeLa cells to heat shock in the presence of SB 203580, the p38 inhibitor (20 μ*M* for 1 h) or PD 98059, the ERK inhibitor (20 μ*M* for 1 h). These compounds do not alter the intracellular localization of DAX-1 protein in cells cultured at 37°C, nor do they affect its redistribution in the cytoplasm subsequent to heat shock (Fig. 3B). We performed control experiments to verify that these inhibitors are active and they are able to block specifically the phosphorylation of p38 and ERK after a proper stimulus (Fig. 3C). We can conclude that the effect of heat shock upon DAX-1 localization is not mediated through the p38 and ERK pathways.

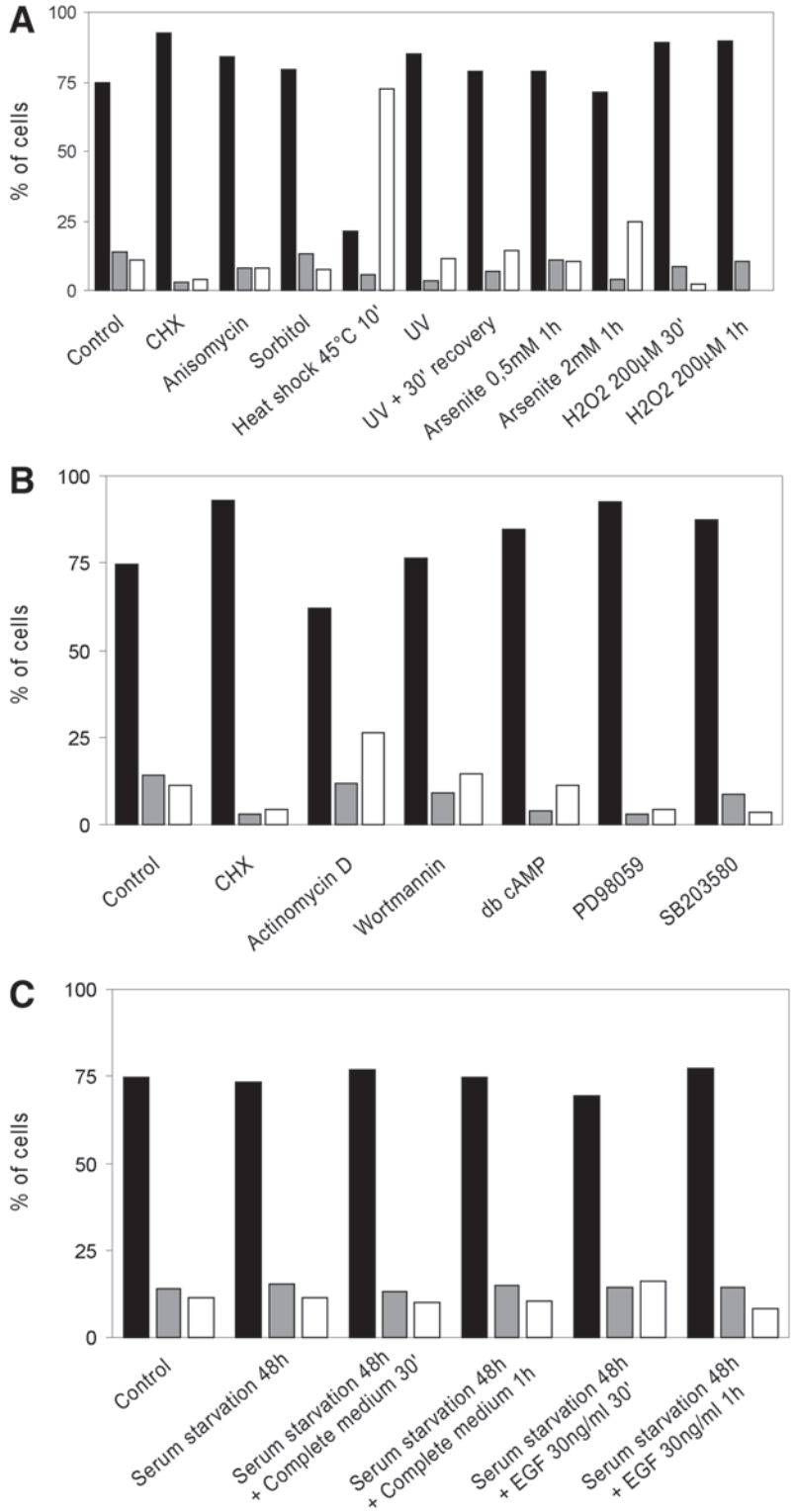

Fig. 1. Subcellular localization of DAX-1 protein in transfected HeLa cells is changed only by heat shock. Percentage of nuclear localization is indicated with black histograms, nucleocytoplasmic localization in grey, and cytoplasmic localization in white. At least 200 transfected cells were scored for each experimental condition. (**A**) Effect of heat shock and other cellular stresses. (**B**) Effect of chemical compounds and inhibitors. (**C**) Effect of culture conditions.

DAX-1 Heat Shock–Induced Nuclear Exclusion Is Reversible

The heat shock–induced cytoplasmic localization is a dynamic and reversible process. Indeed, if we submit cells to a heat shock and then we allow them to recover for 30 min at 37°C, we observe that DAX-1 is again in the nucleus and has the same distribution as in the basal conditions (Fig. 3D). We then wondered if heat-shock-protein 90 (HSP 90) is

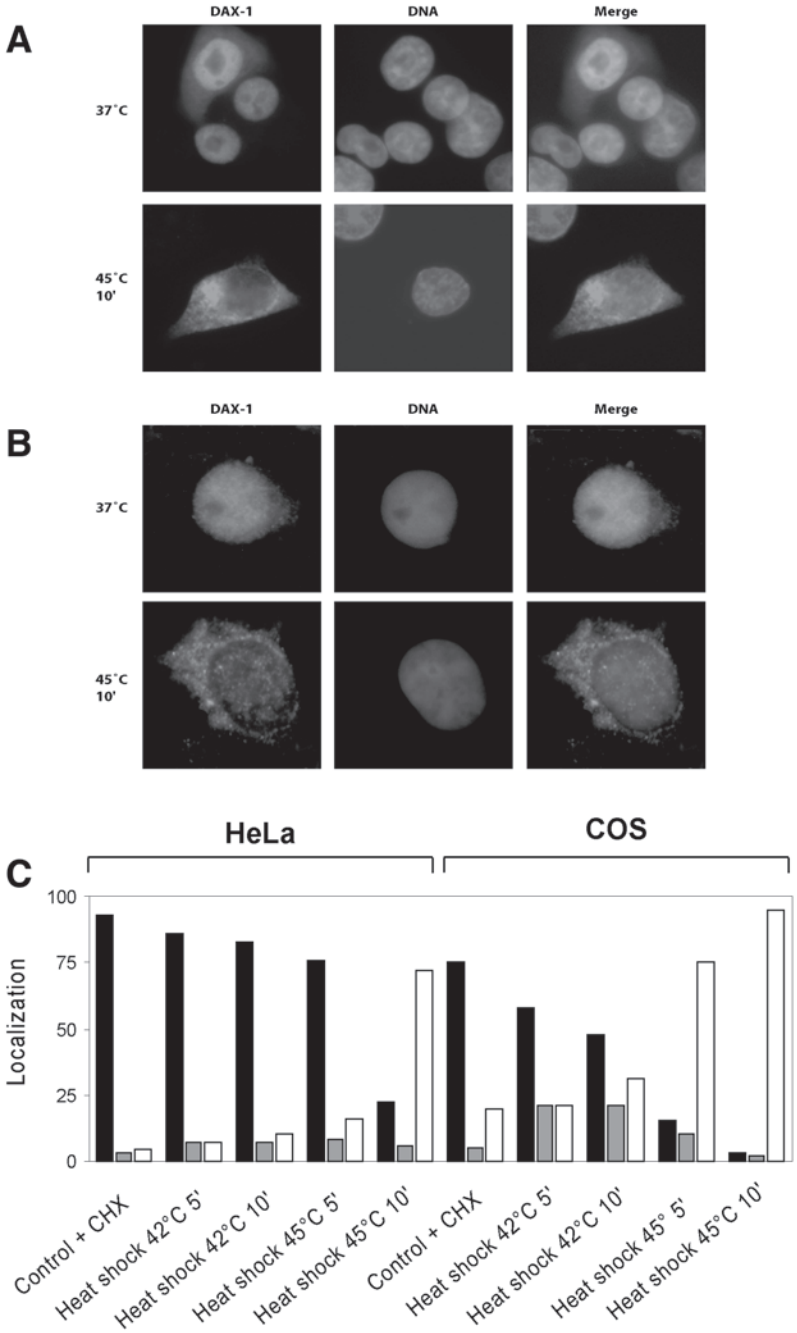

Fig. 2. Heat shock effect is cell type specific. (**A**) HeLa cells growing at 37°C (top) or heat shocked at 45°C for 10 min (bottom). Localization of transfected DAX-1 protein is shown. (**B**) As in (**A**), but localization of the endogenous DAX-1 protein in H295R cells is shown. (**C**) Histogram showing the differential effect of heat shock upon DAX-1 protein distribution in HeLa versus COS cells. Percentage of nuclear localization is indicated with black histograms, nucleocytoplasmic localization in grey, and cytoplasmic localization in white.

involved in DAX-1 changes of localization. We decided to treat the cells with the HSP90 inhibitor geldanamycin and we observed an incomplete recovery of nuclear localization, with 50% of the cells still exhibiting a cytoplasmic localization of DAX-1 30 min after recovery at 37°C. Conversely, HSP90 does not play an essential role for the regulation of DAX-1 subcellular localization in basal conditions, as the treatment of cells with geldanamycin does not affect its subcellular localization in cells cultured at 37°C (Fig. 3D). This observation is consistent with the absence of interaction observed between DAX-1 and components of the heat-shock-protein complex in basal conditions *(10)*.

Heat Shock Increases DAX-1 Insolubility

After heat shock, DAX-1 is located mainly in the cytoplasm and more precisely in the perinuclear region, tending to form aggregates (Fig. 2). We then hypothesized that heat shock acts by affecting DAX-1 solubility, leading to

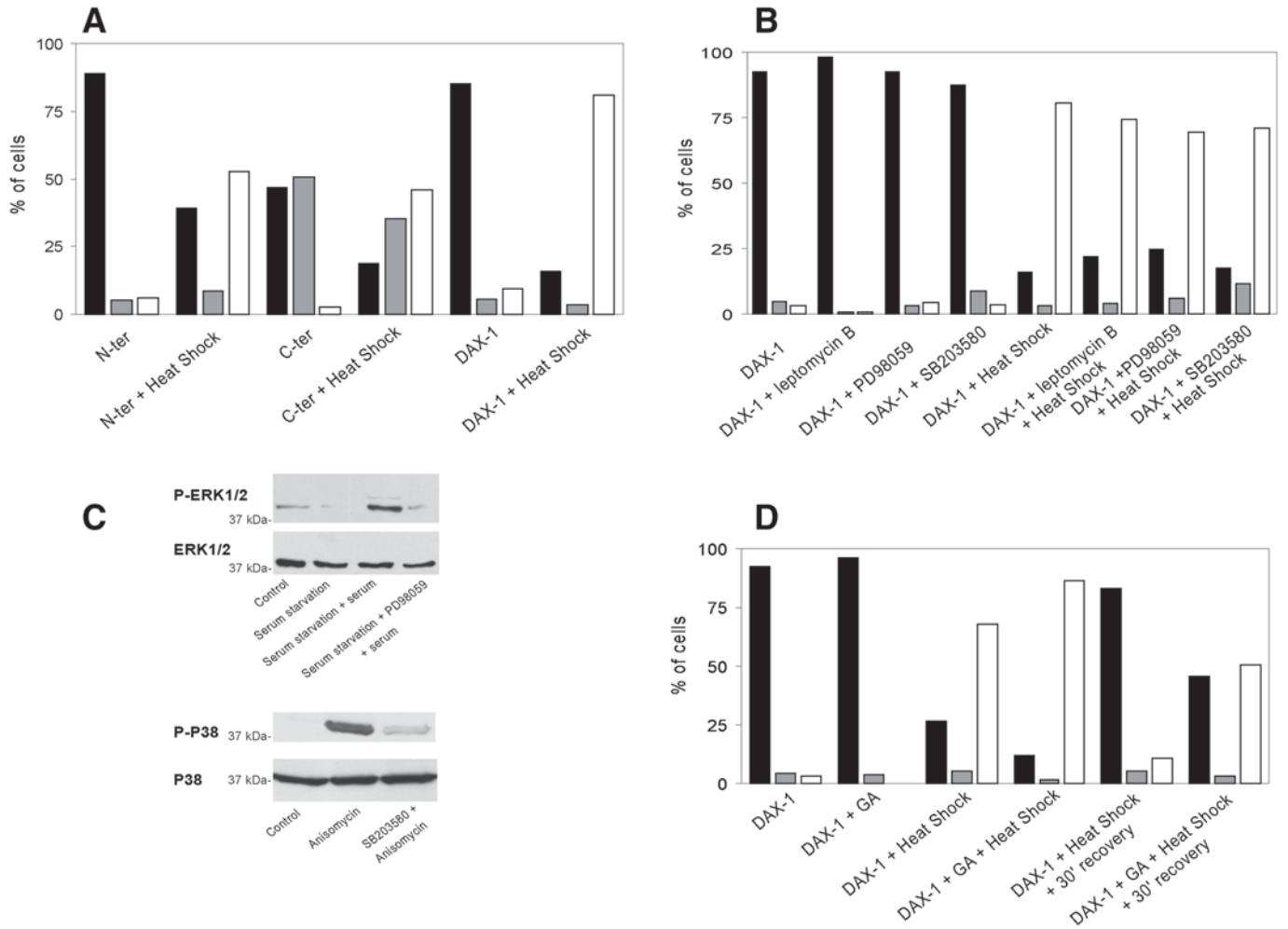

Fig. 3. Effect of drugs upon heat shock effect. (**A**) N- and C-terminal domains of the DAX-1 protein are both sensitive to heat shock. (**B**) Heat shock effect is not sensitive to nuclear export block by leptomycin B, nor to ERK (PD98059) or p38 (SB203580) block. Efficacy of PD98059 and SB203580 is shown in (**C**). (**D**) Heat shock effect is reversible and geldanamycin treatment partially inhibits recovery. In all histograms, percentage of nuclear localization is indicated with black histograms, nucleocytoplasmic localization in grey, and cytoplasmic localization in white.

the formation of aggregates. We analyze the solubility of the DAX-1 protein in a buffer including detergent before, immediately after, and 30 min after a heat shock at 45°C for 10 min. HeLa cells transiently transfected with DAX-1 were lysed in buffer containing 1% Triton X-100. When cells are not submitted to any treatment, DAX-1 is present mainly in the insoluble fraction, but a small percentage (7%) is soluble (Fig. 4). After heat shock, no DAX-1 is found in the soluble fraction. If cells are allowed to recover for 30 min after heat shock, we observe the same distribution as in basal

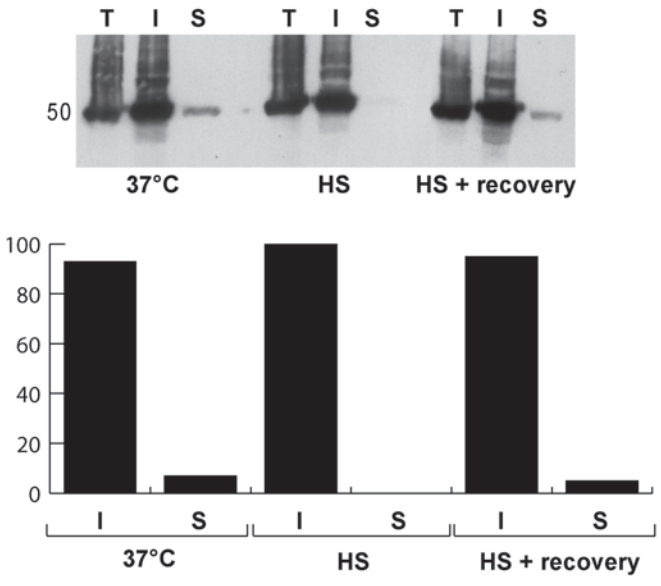

Fig. 4. DAX-1 insolubility is reversibly increased after heat shock. Transfected HeLa cells were extracted with a buffer containing detergent, as described in Materials and Methods, and presence of the DAX-1 protein in soluble (S) and insoluble (I) fractions was assessed by Western blot. Quantification of the results is shown in the histogram.

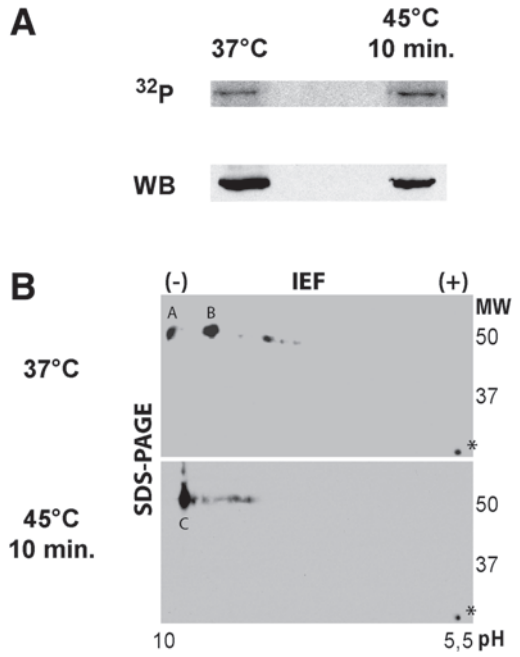

Fig. 5. DAX-1 is modified after heat shock. (**A**) DAX-1 phosphorylation level slightly increases following heat shock. Transfected COS cells were labelled in vivo with ^{32}P-orthophosphate and DAX-1 was subsequently immunoprecipitated in stringent conditions. (**B**) 2D gel electrophoresis of protein extracts from transfected COS cells growing at 37°C (top) or heat shocked at 45°C for 10 min (bottom), showing modifications of the DAX-1 protein, which are changed subsequently to heat shock. The endogenous LDH was used as an internal control (asterisk).

conditions. These data show that heat shock increases the insolubility of DAX-1 and probably leads to aggregation of DAX-1 that is mislocalized in the cytoplasm. This increased insolubility is reversible after recovery at 37°C, and this coincides with the return of DAX-1 to the nucleus.

Heat Shock Induces Modifications of the DAX-1 Protein

The striking observation that heat shock induces a reversible cytoplasmic localization and an increased insolubility of DAX-1 led us to hypothesize that heat shock could induce post-translational modifications of DAX-1 which in turn could modify its solubility. We first checked if DAX-1 phosphorylation level was modified by heat shock. To study this possibility, we performed in vivo orthophosphate labeling of DAX-1 transfected COS-1 cells by incorporation of orthophosphate labeled with ^{32}P to cells cultured in basal conditions or submitted to heat shock and followed by DAX-1 immunoprecipitation. We observed that in basal conditions, DAX-1 is phosphorylated and the level of phosphorylation is slightly increased by heat shock (Fig. 5A).

Next, we performed two-dimensional gel electrophoresis of total protein extracts of COS-1 cells transiently transfected with DAX-1 submitted or not to heat shock. With the Compute pI/Mw tool from the ExPASy web page, we calculated the theoretical pI of DAX-1, which is 8.5. In basal conditions, DAX-1 seems to undergo numerous modifications and be present under different forms of different pI as could be deducted by the presence of numerous dots. We can distinguish two main basic spots A and B that correspond to two major forms, and four smaller spots more acidic than the other two (Fig. 5B). After heat shock, we observed a change of the spots profile, with a single main spot named C, which has a pI intermediate between the pIs of the two main forms observed in basal conditions and probably tree minor spots more acidic, which were not well separated. The endogenous LDH was used as an internal control (asterisk). From these experiments, we can conclude that heat shock induces post-translational modifications of DAX-1 which modify its pI.

Discussion

Nucleocytoplasmic transport constitutes an important aspect of the regulation of fundamental cellular processes, such as gene expression, signal transduction, and cell cycle progression. DAX-1 subcellular localization is modified in AHC and during pituitary development *(10,12)*. It is therefore critical for its function and needs to be tightly controlled. Here we demonstrate that heat shock regulates DAX-1 protein by modifying it, increasing its insolubility and changing its subcellular localization by causing its sequestration in the cytoplasm.

Heat shock induces the phosphorylation and sumoylation of the heat shock factor 1 (HSF1) *(17)*. Our present study indicates that heat shock changes DAX-1 post-translational modifications. As we observed a slight increase in the phosphorylation level after heat shock, we could speculate the heat shock induces phosphorylation. But the change in the two-dimensional electrophoresis spots profile cannot be explained only by phosphorylation. We then speculate that other modifications such as acetylation, glycosylation, methylation, sumoylation, or ubiquitination may occur in response to heat shock. Because no post-translational modifications of DAX-1 have been reported up to date, the characterization of these modifications and their changes by heat shock would be essential for understanding the regulation of DAX-1 function.

Here, we show that heat shock reversibly increases DAX-1 insolubility and leads to the formation of aggregates. This phenomenon is also seen with several proteins like luciferase *(18)*, MKP-1 and 3 ERK phosphatases *(16)*, and M3/6 JNK phosphatase *(19)*. In all these cases and also in the case of DAX-1, heat-induced inactivation is reversible under the conditions of heat shock performed. The severity of the heat shock–induced damage is critical for determining the functional restoration of the aggregated protein via some chaperones as the ClpB/Hsp104 and Dna K/HSP 70, or its targeting to proteolysis *(20)*. In the case of DAX-1, the chaperone HSP90 seems to be important for the recovery after

heat shock of the nuclear localization and probably the restoration of the aggregates, but other factors may be implicated.

DAX-1 heat shock–induced cytoplasmic localization is reversible and requires the cooperation of the N- and C-terminal domains and is independent of Crm1, p38, and ERK. The heat shock effect on DAX-1 localization is highly specific because other stimulations inducing cellular stress (sorbitol, UV, arsenite, H_2O_2) and the activation or inhibition of diverse pathways do not affect it. Heat shock induces a strong stress response and affects the nucleocytoplasmic distributions of a variety of proteins such as importinα 70 kD heat shock cognate protein (hsc70) *(21)*, CK2 protein kinase *(22)*. Nevertheless, the effect of heat shock on importinα trafficking is not specific, because other cellular stresses produce the same effect *(23)*. Interestingly, another member of the nuclear receptor family, the glucocorticoid receptor, behaves differently and in its unliganded state moves to the nucleus after heat shock *(24)*. It would be interesting to test the behavior of other members of the family in response to heat shock.

It has been shown that heat shock reversibly inhibits the binding of a protein complex from MA10 cells (mouse Leydig tumor cells) to the StAR promoter *(25)*. As DAX-1 binds to StAR promoter *(8)*, and considering the multiple effects of heat shock on DAX-1, we can speculate that subsequent to heat shock, the ability of DAX-1 to bind to protein partners and/or to DNA is impaired. In addition, we have recently shown that subcellular DAX-1 localization in the human fetal adrenal gland is modulated by the composition of the extracellular matrix and by hormonal stimulation with ACTH and angiotensin II, two important regulators of steroid hormone production *(26)*. It is then tempting to speculate that heat shock may mimic physiological conditions that drive DAX-1 localization in the human fetal adrenal gland.

Materials and Methods

DNA Clones

pSV.DAX-1, pSV.DAX-1 R3 (1-205), and pSV.LBD (205-470) expression vectors *(10,11)* were used for transfection experiments.

Cell Culture and Transfection

HeLa cells were cultured in DMEM containing 2.5% calf serum, 2.5% FCS, and gentamycin. COS-1 cells were cultured in DMEM containing 5% FCS and gentamycin. Human adrenocortical H295R cells were cultured in F12-Dulbecco's modified Eagle's medium supplemented with 2% Nu-Serum (Collaborative Research), 1% ITS Plus (Collaborative Research), 15 m*M* Hepes, and penicillin–streptomycin. Cells were seeded into six-well plates (3×10^5 cells/well) and transfected by the calcium phosphate method. For the different stimulations, cells were treated with different inhibitors at the concentrations and times indicated below. For heat shock, the cells, seeded on tissue culture plates, were floated in a 45°C water bath for 10 min. The heat shock treatment did not affect cell viability, as measured by the Trypan blue method.

Immunofluorescence

Immunofluorescence was performed as previously described *(10)*. Briefly, transfected HeLa cells growing in chamber slides (Nunc, France) were fixed 15 min with 4% paraformaldehyde, permeabilized 20 min with 0.1% Triton X-100 in PBS, blocked 30 min with 2% BSA in PBS, and incubated overnight with the 2E5 monoclonal antibody, which recognizes an epitope in the DAX-1 N-terminal domain, to detect full-length DAX-1 and its N-terminal domain, or the 3E5 monoclonal antibody to detect the DAX-1 C-ter domain. After washing 20 min with 0.1% Triton X-100 in PBS, the primary antibody was revealed by incubation for 1 h with an Alexa 594-labeled goat anti-mouse secondary antibody (Molecular Probes, The Netherlands). DNA was counterstained with Hoechst 33342 dye. Subcellular localization was scored as nuclear when more than 90% of the protein was present in the nucleus. It was scored as cytoplasmic when more than 90% of the protein was present in the cytoplasm and nucleocytoplasmic in all other cases. Data were computed from at least two experiments, with each experiment scoring at least 200 transfected cells.

Solubility Assays

For determination of DAX-1 solubility, transiently transfected HeLa cells were scraped and lysed in buffer containing 40 m*M* Hepes pH 7.9, 50 m*M* KCl, 1% Triton X-100, 5 m*M* EDTA, 2 m*M* DTT, 1 m*M* PMSF, and supplemented with a protease inhibitor cocktail. Supernatant (named as soluble fraction) and pellet (insoluble fraction) fractions were separated by centrifugation at 12,000*g* for 10 min and analyzed by SDS-PAGE and Western blot using the 2E5 monoclonal antibody to detect DAX-1.

In Vivo Orthophosphate Labeling

Transiently transfected COS-1 cells were incubated in DMEM without orthophosphate for 30 min, and thereafter with DMEM containing orthophosphate labeled with ^{32}P (Amersham, France; 1 mCi/mL) for 3 h. The cells were either subjected or not to heat shock and harvested, lysed in 200 μL boiling Buffer I (50 m*M* Tris pH 8, 0.5% SDS) and scraped. Eight hundred microliters ice-cold RIPA buffer (20 m*M* Tris pH 7.4, 150 m*M* NaCl, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 5 m*M* EDTA, phosphatase inhibitor cocktail, 0.5 m*M* DTT, 0.5 m*M* PMSF) were then added and immunoprecipitation of DAX-1 using the 2E5 antibody was performed. Immunocomplexes were captured on protein G Sepharose (Pharmacia, France) and washed four times with 0.5 mL Buffer II (50 m*M* Tris pH 7.4, 150 m*M* NaCl, 0.5% Nonidet P-40, phosphatase inhibitor cocktail, 0.5 m*M* DTT, 0.5 m*M* PMSF) at 4°C. Proteins on beads were eluted with Laemmli buffer and run on a SDS-PAGE

gel. This was dried and exposed to X-Omat (Kodak, France) film for autoradiography.

Two-Dimensional Gel Electrophoresis

Transiently transfected COS-1 cells were resuspended and lysed 10 min in isoelectric focusing (IEF) buffer (7 *M* urea, 2 *M* thiourea, 2% CHAPS, 50 m*M* DTT). The cell lysate was cleared by centrifugation at 10,000*g* for 10 min. Biolytes (pH 3–10) (Biorad) were added to the extract (0.2%) and 200 µg of total proteins were loaded on ReadyStrips (pH 3–10) and allowed to rehydrate during 9 h. A step gradient of 50–8000 V was applied to the strips followed by constant 8000 V, with focusing complete after 60,000 V-h. The protean IEF cell BioRad apparatus was used for the IEF. The strips were then incubated 20 min in equilibration buffer (6 *M* urea, 0.375 *M* Tris pH 8.8, 2% SDS, 20% glycerol, and 2% DTT) and inserted onto a 12% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and probed with the 2E5 antibody *(13)* to visualize DAX-1 or with a mouse anti-LDH antibody (Sigma) for the endogenous LDH.

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