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Research Article

Dissection of the molecular mechanisms that control the nuclear accumulation of transport factors importin-α and CAS in stressed cells

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Abstract. The physiological state of eukaryotic cells controls nuclear trafficking of numerous cargos. For example, stress results in the inhibition of classical protein import, which is characterized by the redistribution of several transport factors. As such, importin-α and cellular apoptosis susceptibility protein (CAS) accumulate in nuclei of heat-shocked cells; however, the mechanisms underlying this relocation are not fully understood. We now show that heat upregulates the initial docking of importin-α at the nuclear envelope and stimulates the translocation of CAS into the nuclear interior. Moreover, heat exposure compromises the exit of importin-α from nuclei and drastically increases its retention in the nucleoplasm, whereas CAS nuclear exit and retention are less affected. Taken together, our results support the idea that heat shock regulates importin-α and CAS nuclear accumulation at several levels. The combination of different stress-induced changes leads to the nuclear concentration of both transport factors in heat-stressed cells.

Keywords. Stress, heat shock, nucleus, transport.

In eukaryotic cells, transport of proteins and RNA in and out of the nucleus is mediated by nuclear pore complexes (NPCs), located at the junctions of inner and outer nuclear membranes. In addition to nucleoporins, many of these transport processes require soluble factors [1]. Nucleocytoplasmic trafficking of macromolecules is essential to maintain cellular homeostasis and is therefore subject to multifactorial regulation. This regulation may take place at the level of individual cargos or the transport apparatus by affecting either NPCs or soluble transport factors. For example, altered NPC function may modulate the passage of all macromolecules across the nuclear envelope (NE) [2, 3], and changing the activity or abundance of nuclear carriers can control transport of multiple cargos [4–6].

Classical nuclear import is mediated by a carrier system that moves numerous proteins into the nucleus, as the dimeric receptor importin-α/importin-β1 translocates proteins with classical nuclear localization signals (NLSs) across the NPC. Upon completion of the import reaction, both subunits of the classical import receptor return to the cytoplasm. Export of importin-α to the cytoplasm depends on cellular apoptosis susceptibility protein (CAS), a carrier of the importin-β family, and RanGTP [7, 8]. In addition to its crucial function as a nuclear exporter, CAS

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participates in mitotic spindle checkpoint control, contributes to proper development and was recently identified as a regulator of p53-dependent transcription [5, 9, 10]. Like CAS, importin-α has multiple functions that are not limited to nuclear transport [11, 12]. Importin-α traverses the NE while bound to importin-β1 or CAS; however, it also translocates into the nucleus in the absence of other factors, a pathway that is poorly defined [13].
Multiple physiological and pathophysiological states control the movement of macromolecules in and out of the nucleus [14, 15]. These include the age-dependent loss of nuclear transport efficiency in human fibroblasts which correlates with reduced levels of importin-α, CAS and RanBP1 [6]. Stress in particular regulates the movement of numerous classical and non-classical cargos. Different stressors exert a general control on nuclear trafficking by affecting several components of the transport machinery. As such, we and others have shown that the steady-state concentration of importin-α and CAS increases in nuclei of stressed cells [16, 17]. At present, the mechanisms that contribute to this relocation are not fully understood. A combination of several dynamic processes determines the steady-state distribution of macromolecules that shuttle between the nucleus and the cytoplasm. These include nuclear import, export and retention within the nuclear or cytoplasmic compartment. Each of these processes can make different contributions to the steady-state localization of a protein, and their input may adapt to changes in cell physiology. *In vitro* assays are particularly useful to dissect nuclear trafficking and evaluate the role of individual trafficking steps [18]. Here, we show that heat shock stimulates the nuclear accumulation of importin-α and CAS in a cell-free system, and we define several mechanisms that concentrate importin-α and CAS in nuclei *in vitro* and in growing cells. Our studies identify multiple heat-sensitive steps that contribute differently to the stress-induced nuclear accumulation of importin-α and CAS.

Materials and methods

Growth of HeLa cells and heat shock. HeLa cells were grown on multiwell chambers or cover slips following published procedures [19]. For heat treatment, cells at 50–70 % confluence were incubated for 30 min at 45.5 °C. All of the results were obtained multiple times, with at least three independent experiments for each data point.

Immunofluorescent staining. All steps were carried out at room temperature, essentially as described elsewhere [20]. Antibodies against Ran (1:200), importin-α (1:200), CAS (1:100), all from Santa Cruz Biotechnology (Santa Cruz, CA), were diluted as indicated. Three different treatments were used for staining nuclear, cytoplasmic proteins or both (summarized in Fig. 1). For treatment 1, cells were fixed first and all membranes permeabilized with Triton X-100. This protocol monitors the protein distribution in all cellular compartments. Treatment 2 locates transport factors in the cytoplasm, including the cytoplasmic side of the nuclear envelope. To this end, cells were first fixed and then treated with digitonin to permeabilize the plasma membrane; all subsequent steps were carried out without detergent to prevent access of antibodies to the nuclear interior. The NE remains intact under these conditions (see Fig. 2A). Treatment 3 visualizes nuclear proteins and cytoplasmic proteins that were not solubilized by digitonin extraction. For this treatment, unfixed cells were incubated with digitonin. Following digitonin extraction, samples were fixed and nuclear membranes were permeabilized with Triton X-100. For treatment 1 and 3, after Triton X-100 permeabilization, samples were blocked in PBS/2 mg/ml BSA/0.05 % Tween 20 (PBS/BSA/Tween) for 1 h, followed by overnight incubation with primary antibodies, diluted in PBS/BSA/Tween. Samples were washed three times in PBS/BSA/Tween and primary antibodies were detected with Cy3-conjugated secondary antibodies (1:500, 2 h; Jackson ImmunoResearch, West Grove, PA). After three washes with PBS/BSA/Tween, DNA was visualized with 4',6-diamidino-2-phenylindole (DAPI) and samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA). For treatment 2, there was no permeabilization with Triton X-100, and Tween 20 was omitted from all steps of the procedure. Mounted samples were analyzed with a Nikon Optiphot at ×400 magnification and photographed with Kodak T-MAX 400 films or with a Zeiss LSM 510 (×63 objective, 1.4 NA). Images were processed with Photoshop 5.5 and 8.0.

Western blot analysis. Western blotting and ECL followed standard procedures [21]. In brief, HeLa cells were grown on dishes to 50–70 % confluency. Control and heat-stressed cells were washed with PBS and stored at −70 °C until use. Crude extracts were prepared by solubilizing proteins in gel sample buffer, pH 8.0, containing protease inhibitors (aprotinin, leupeptin, pepstatin, each at 1 μg/ml, 1 mM PMSF), 20 mM β-glycerophosphate, 1 mM NaN_3, 2.5 mM NaF. Samples were incubated for 10 min at 95 °C and vortexed with glass beads to shear DNA. After centrifugation (5 min, 13 000 rpm, microfuge), aliquots were separated on the same gel. Protein was

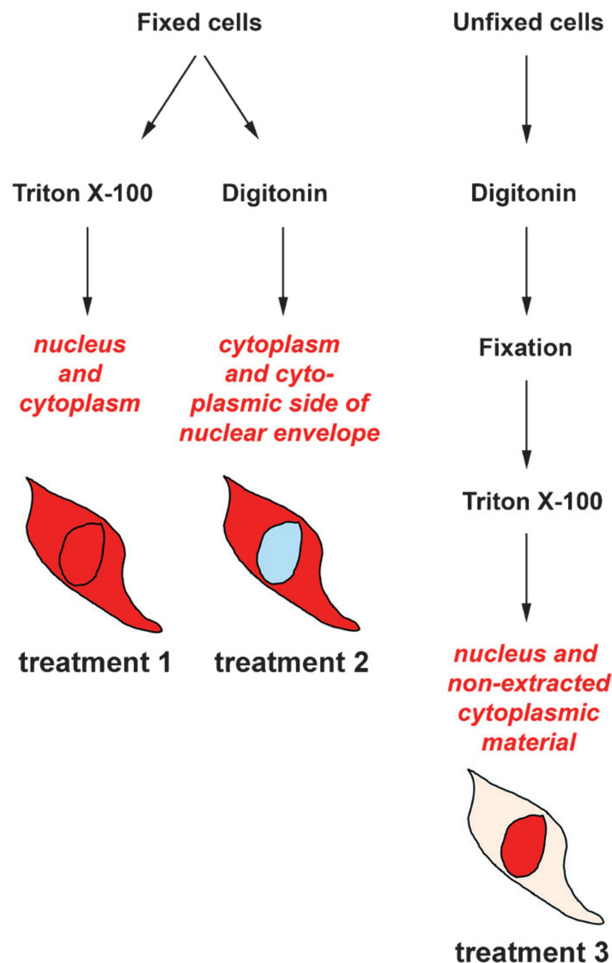


Figure 1. Processing of growing cells to detect proteins in nuclear and cytoplasmic compartments. The different treatments used to localize transport factors in the nucleus and cytoplasm are summarized. Material preferentially stained upon each treatment is shown in red. Treatment 1 localizes proteins in both nuclei and the cytoplasm. Treatment 2 stains material in the cytoplasm, including the cytoplasmic side of the nuclear envelope. Treatment 3 will detect antigens in the nucleus and cytoplasmic material that is resistant to digitonin extraction. See text for details.

stained with Coomassie and quantified by densitometry using Spot density tools software as recommended by Alpha Innotech Corporation (San Leandro, CA). For Western blotting, equal amounts of protein as determined by densitometry were separated by SDS-PAGE, transferred to nitrocellulose and incubated overnight with primary antibodies at 4 °C. Antibodies were used at the following dilutions: importin-α1 (1:500, sc-6917; Santa Cruz Biotechnology), importin-β1 (1:400, sc-11367), CAS (1:200, sc-1708), Ran (1:500, sc-1156), tubulin (1:2,000, sc-5286) and actin (1:1,000; Chemicon, Temecula, CA). Washed filters were incubated with HRP-conjugated secondary antibodies, washed and processed for ECL (GE Healthcare).

Classical nuclear import. Generation of the classical nuclear import substrate SV40-HSA, labeling and *in vitro* nuclear import have been described previously [22]. HeLa cells grown to 50–70% confluency were exposed to 45.5 °C for 30 min. Control and heat-shocked cells were permeabilized with 40 μg/ml digitonin in buffer B (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT) and 1 μg/ml of protease inhibitors [aprotinin, leupeptin and pepstatin or protease inhibitor cocktail (Roche, Basel, Switzerland)] followed by incubation with 50 μg/ml fluorescent substrate in buffer B containing 10 mg protein/ml of cytosol, 1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine kinase. After 30 min incubation at 30 °C, cells were washed with buffer B and fixed for 10 min with 3.7% formaldehyde in PBS at room temperature. Slides were rinsed with PBS, incubated with 1 μg/ml DAPI and mounted.

Purification of proteins synthesized in *Escherichia coli* and fluorescent labeling. Tagged proteins were synthesized in *E. coli* and purified under native conditions following standard procedures. Purified importin-α and CAS were labeled with Oregon Green 488 iodoacetamide or tetramethylrhodamine-maleimide (TMR; Molecular Probes) as recommended by the supplier. Covalent modification of sulfhydryl groups has been used successfully to study *in vitro* nuclear trafficking of importin-α, CAS and other nuclear transport factors [17, 23, 24]. In brief, purified proteins were dialyzed against 25 mM sodium phosphate pH 7.1 and centrifuged for 3 min at 13 000 rpm (microfuge). Oregon Green 488 iodoacetamide or TMR was dissolved in N,N-dimethyl formamide and a fivefold molar excess was added to the protein. Upon overnight incubation on ice, non-incorporated dye was removed by gel exclusion chromatography and aliquots of the labeled import substrates were stored at –70 °C.

***In vitro* nuclear import of importin-α and CAS.** Import of TMR-labeled importin-α or CAS was analyzed in semi-intact HeLa cells at a final concentration of 400 nM in buffer B [17, 25]. Import assays containing 1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine kinase were supplemented with 3 mg/ml cytosol prepared from control or heat-shocked HeLa cells (30 min, 45.5 °C). After 5 min at 30 °C, samples were fixed and stained with DAPI. Fluorescently labeled transport factors were localized by confocal laser microscopy and intranuclear fluorescence was quantified as described below; for each condition 54–59 cells were scored.

Docking of importin-α and CAS at nuclear envelopes. Docking at the NE was tested with fluorescent proteins; all steps were carried out on ice. HeLa cells were washed in cold buffer B and incubated with 40 μg/ml digitonin in buffer B for 5 min. Cells were washed once with buffer C (buffer B without DTT and protease inhibitors) and extracted for 10 min with buffer B containing 1 M NaCl and 3 mg/ml BSA. Samples were rinsed twice with buffer B/BSA and incubated with fluorescently labeled importin-α (80 μg/ml) or CAS (160 μg/ml) for 10 min. After rinsing twice with buffer B/BSA and once with PBS, samples were fixed for 10 min in 3.7 % formaldehyde in PBS and nuclei were stained with DAPI. Binding of fluorescent substrates was quantified for 43–65 cells with the Multiwavelength Translocation Module (see below).

Nuclear exit of importin-α and CAS *in vitro*. Unstressed or heat-shocked HeLa cells were semi-permeabilized by a 5-min treatment with digitonin in buffer B, followed by incubation at 37 °C with transport buffer supplemented with an energy-regenerating system, 1 mM GTP and 50 μg/ml RanQ69L, a Ran mutant that mimics RanGTP. Samples were fixed after 5, 10 and 15 min. Controls (0 min) were fixed immediately after digitonin extraction. Importin-α and CAS were detected by immunofluorescent staining, followed by quantification of the fluorescence intensities. For each condition between 48 and 75 cells were quantified.

Measurement of intranuclear and nuclear envelope fluorescence. Intranuclear and nuclear envelope fluorescence were obtained by optical sectioning with a Zeiss LSM 510 laser scanning confocal microscope, using a NA 1.4 × 63 objective [17]. Image analysis was performed with Metamorph or MetaXpress software by adaptation of the Translocation Enhanced or Multiwavelength Translocation modules. Nuclei were identified as 100–350 μm^2 area with a width of ~10 μm and an intensity of DAPI staining over local background of > 5 intensity units. The segmentation region defined by DAPI staining was then used to measure pixel intensities of the Oregon Green or tetramethylrhodamine images. All images were corrected for the contributions of background intensity using regions of the images that did not contain cells. Average pixel intensities for nuclear or nuclear envelope areas were calculated for individual cells. All segmentation data sets were inspected manually to ensure accuracy of the data, and cells with inaccurate segmentation were excluded from the analysis. For each experimental condition, pixel intensities were measured for 43–75 cells that showed accurate segmentation.

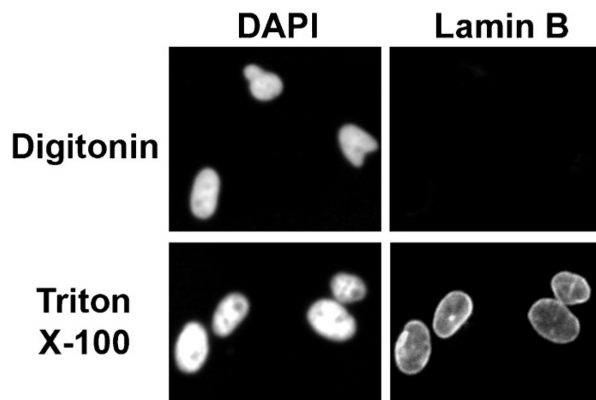
Statistics. To measure fluorescence signals in nuclei and cytoplasm or docking at the nuclear envelope, data for 43–75 cells that showed accurate segmentation were acquired for each of the different conditions. Results are shown as means ± SD; statistical analyses were carried out essentially as described elsewhere [21].

Nuclear retention of transport factors. Nuclear retention in *unfixed* cells was monitored by extraction with KM buffer (10 mM N-morpholinoethanesulfonic acid, pH 6.2, 10 mM NaCl, 1.5 mM $MgCl_2$, 10 % glycerol, protease inhibitors), buffer containing detergent (Nonidet P-40; NP40), DNase, NaCl, DNase + RNase essentially as described previously [26]. Samples were fixed after each step and processed for immunofluorescent staining and confocal microscopy. This extraction protocol has been developed for the preparation of nuclear matrices [26]. The bulk of cellular protein (~60 %) is solubilized by treatment with NP40 at low ionic strength, whereas components of the cytoskeleton and several nuclear compartments are resistant to this treatment [26]. Subsequent incubation with DNase releases about 4 % of the protein, without drastically affecting nucleoli or the cytoskeleton. High salt removes about 29 % of the protein, including a portion of the cytoskeleton and histones. After the final DNase/RNase step, samples contain the nuclear matrix, remnants of nucleoli and the lamina. This material, resistant to all of the extraction steps, is equivalent to ~10 % of the cellular protein [26].

Results

Heat stress leaves nuclear envelopes intact and inhibits classical nuclear import *in vitro*. Various stresses, including heat shock, interfere with nuclear transport in growing cells, and we examined whether this can be recapitulated in a cell-free system. To this end, HeLa cells were pre-incubated for 30 min at 45.5 °C and subsequently treated with digitonin to permeabilize the plasma membrane. The NE remained intact under these conditions, and lamin B was not accessible to antibodies. However, antibodies bound when nuclear membranes were permeabilized with Triton X-100 (Fig. 2A). The effect of heat on classical nuclear import *in vitro* was monitored with digitonin-permeabilized cells, which were supplied with TRITC-labeled SV40-HSA as import substrate, energy and unstressed cytosol [22]. SV40-HSA efficiently translocated into nuclei of unstressed cells, where it concentrated in nucleoli. By contrast, heat-shocked semi-intact cells failed to accumulate the classical

import cargo in nuclei (Fig. 2B), supporting the idea that upon heat shock, semi-intact cells reiterate faithfully classical import inhibition. In the experiments described below, this *in vitro* system was used to analyze trafficking of nuclear transport factors.



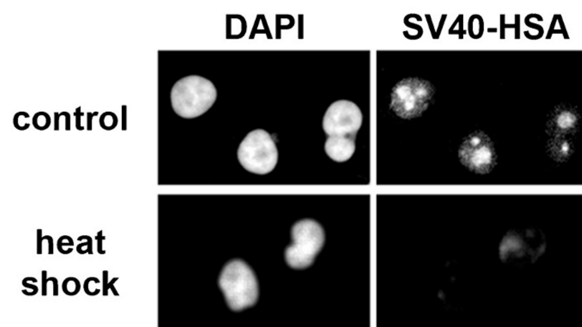


Figure 2. (*A*) NEs remain intact in heat-stressed cells. HeLa cells exposed to 30 min at 45.5 °C were treated with digitonin or Triton X-100 and incubated with antibodies against lamin B. After 1.5 h at 30 °C, cells were fixed, and binding of anti-lamin B antibodies was examined with FITC-conjugated secondary antibodies. Lamin B was not accessible to antibodies when cells were treated with digitonin only. However, antibodies bound to lamin B if nuclear membranes were permeabilized with Triton X-100. (*B*) Heat shock inhibits classical nuclear import *in vitro.* Classical nuclear import was analyzed with TRITC-SV40-HSA in semi-intact control and heat-shocked HeLa cells.

Heat stress relocates importin-α, CAS and Ran, but does not change transport factor abundance. The experiments in Figure 3A address several questions. First, we compared the distribution of import factors in whole cells (treatment 1) that were kept under normal growth conditions (Fig. 3A, control) or exposed to heat (heat shock). Furthermore, import factor levels in the nucleus (treatment 3) or cytoplasm (treatment 2) were monitored.

In intact cells (Fig. 3A, treatment 1) upon heat shock, significant changes were seen for Ran, importin-α and CAS. Ran partially relocated to the cytoplasm, whereas importin-α and CAS accumulated in nuclei. Treatment 3 was used next to determine the effect of heat stress on *nuclear* transport factors. Although digitonin extraction (Fig. 3A, treatment 3) of unfixed cells solubilized a portion of Ran in control and stressed samples, Ran was still detectable in nuclei. A more pronounced effect of heat shock was detected for the distribution of importin-α and CAS. After digitonin extraction, higher amounts of importin-α and CAS remained associated with heat-shocked nuclei compared to unstressed controls. These results were consistently observed in multiple experiments.

Interestingly, digitonin extraction affected the distribution of nuclear importin-α in control cells (compare treatment 1 with 3); it changed from predominantly nuclear rim localization to a distribution throughout the nucleus in digitonin-treated cells. One possible explanation for this relocation could be a labile association of importin-α with the nuclear envelope which is sensitive to digitonin treatment.

Treatment 2 monitors nuclear transport factors in the cytoplasm of control and stressed cells (Fig. 3A). Heat shock increased the levels of Ran in the cytoplasm, but diminished the cytoplasmic amounts for importin-α or CAS. These results are consistent with the staining pattern obtained for whole cells (Fig. 3A, treatment 1). Together, the data support the idea that stress increased the nuclear, but reduced the cytoplasmic concentration of importin-α and CAS. The opposite scenario applies to Ran.

To determine the effect of heat stress on the abundance of nuclear transport factors, crude extracts were prepared from control and heat-shocked cells. Equal amounts of protein were analyzed side-by-side by Western blotting, with tubulin and actin serving as controls. As shown in Figure 3B, no drastic changes for the levels of Ran, importin-α or CAS were detected in stressed cells.

Heat shock affects nuclear accumulation of importin-α and CAS *in vitro*. Importin-α can enter the nucleus by two pathways, either independent or dependent on cytosolic factors [13]. Figure 4 compares the nuclear accumulation of fluorescently labeled importin-α or CAS in the absence or presence of exogenously added cytosol. Cytosol was prepared from either unstressed or heat-shocked cells and combined with control or heat-treated semi-intact HeLa cells. In the absence of exogenous cytosol, importin-α and CAS entered the nucleus and heat shock stimulated this process significantly (Fig. 4A, C, cytosol –). These results are consistent with the idea that heat stress impinges on

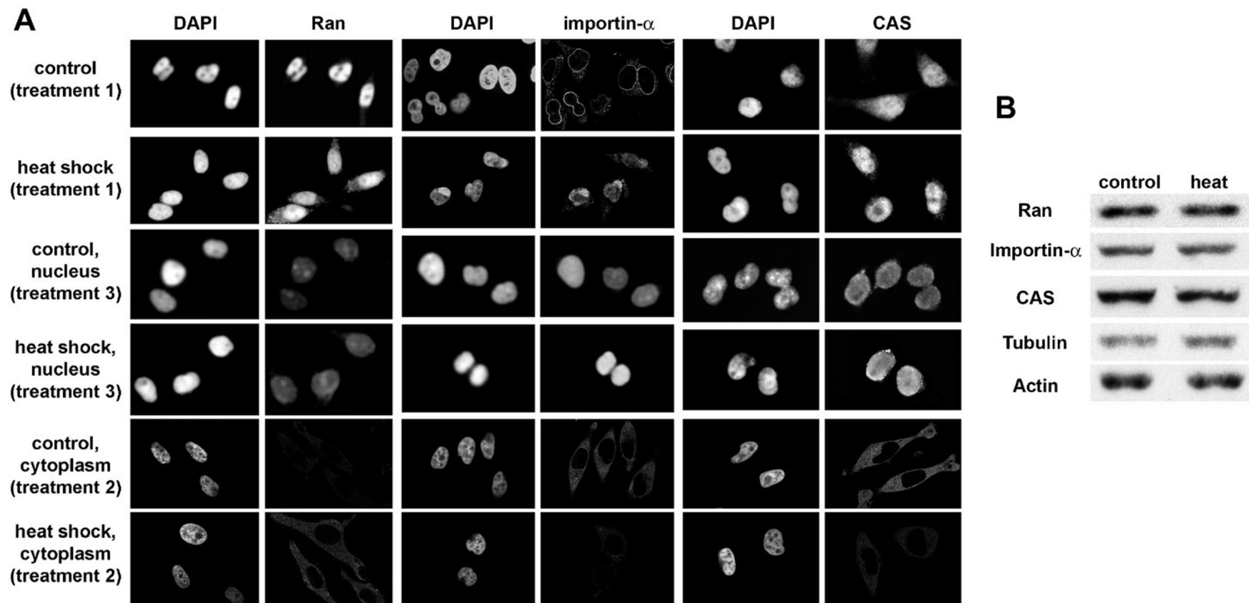


Figure 3. (*A*) Effect of heat stress on the distribution of nuclear transport factors. HeLa cells were grown at 37 °C (control) or stressed for 30 min at 45.5 °C (heat shock). Cells were either fixed immediately (treatment 1; see Fig. 1 for details) or treated with digitonin *before* fixation to stain preferentially nuclear protein (treatment 3). Alternatively, to visualize transport factors located in the cytoplasm (treatment 2), samples were fixed first, followed by permeabilization of the plasma membrane with digitonin. Note that importin-α and CAS concentrate in the nuclei of cells that have been exposed to heat, whereas little protein is detected in the cytoplasm. (*B*) Effect of heat stress on the abundance of Ran, importin-α and CAS. Crude extracts from control and heat-shocked (heat) cells containing equal amounts of protein were analyzed by Western blotting with antibodies against Ran, importin-α or CAS. Actin and tubulin were included as controls. All of the data are representative of at least three independent experiments with similar results.

semi-intact cells, possibly altering nucleoporins or other components of the NE.

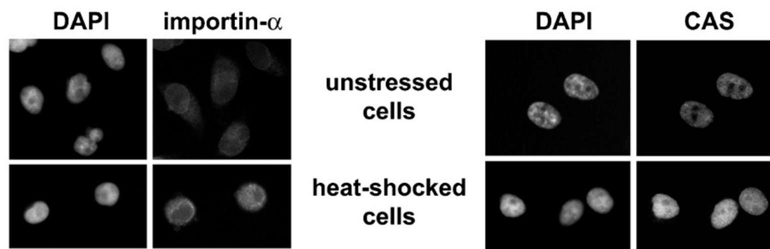
A more complicated picture emerged for importin-α when cytosol was added to *in vitro* assays. The combination of heat-shocked cells (hs) with unstressed cytosol (un) led to a reduction of import when compared with import for unstressed cells/unstressed cytosol (Fig. 4C). However, in heat-shocked semi-intact cells, stressed cytosol significantly enhanced the nuclear accumulation of importin-α when compared with unstressed cytosol (Fig. 4C, xx). This suggests that heat treatment affected components of semi-intact cells as well as cytosolic factors, and both controlled nuclear transport of importin-α. For the carrier CAS, the highest nuclear accumulation was observed with heat-shocked cells/heat-shocked cytosol, and CAS nuclear import was significantly increased with respect to unstressed cells/unstressed cytosol (Fig. 4C, **) or stressed cells/unstressed cytosol (Fig. 4C, xx). Similar results were obtained for several independent experiments, as described in Materials and methods.

Binding of importin-α and CAS to nuclear envelopes *in vitro*. Results in the previous section demonstrated that the *in vitro* nuclear accumulation of importin-α and CAS was modulated by heat shock. Docking at the NE is the initial step required for nuclear import, and we tested whether this process was altered by heat (Fig. 5). Two different experiments addressed this question. First, semi-intact cells were incubated with fluorescently labeled importin-α or CAS, and their association with the NE was quantified (see Materials and methods). This assay measures the sites at the NE that are *free* to bind either transport factor. Second, semi-intact cells were extracted with salt to remove endogenous transport factors associated with nuclear membranes. Subsequent incubation with labeled importin-α or CAS measures the *total* binding sites at the NE that can be occupied.

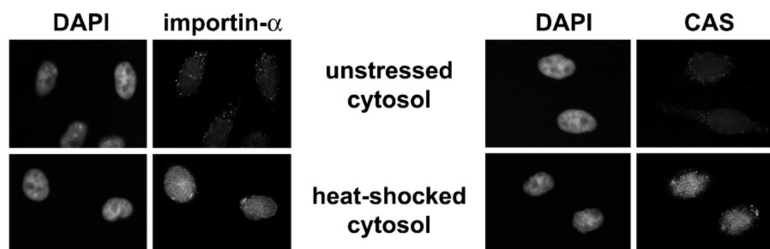
Using these strategies, quantification of fluorescent signals at the nuclear periphery showed that docking of importin-α was increased upon heat stress. This applied both to free and total binding sites (Fig. 5B); more binding sites at the NE became available upon heat shock without (free binding sites) and with (total binding sites) salt extraction. A distinct scenario emerged for CAS, for which we did not detect a significant effect of heat on either free or total binding sites, suggesting that docking of importin-α and CAS may be regulated differently.

For both importin-α and CAS, extraction with salt increased the available binding sites in unstressed and heat-shocked cells (Fig. 5, compare –NaCl with

A ***In vitro*** **import, no cytosol added**



B ***In vitro*** **import, heat-shocked cells, cytosol added**



C **Mean intranuclear fluorescence** **, xx P < 0.001; * P = 0.01

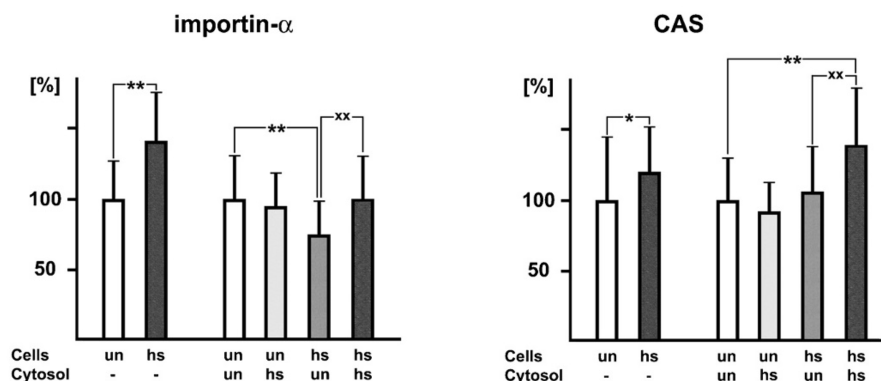
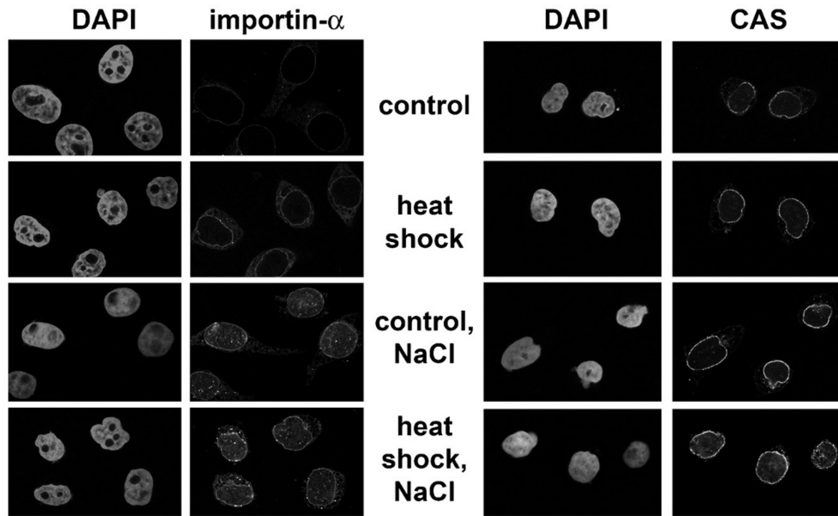


Figure 4. Heat stress alters the nuclear accumulation of importin-α and CAS *in vitro.* Nuclear import of fluorescently labeled importin-α or CAS was analyzed in unstressed and heat-shocked cells in the absence of exogenous cytosol (*A*) or upon addition of cytosol prepared from unstressed or heat-treated HeLa cells (*B*). (*C*) The mean intranuclear fluorescence was measured for each of the different conditions (see Materials and methods). In the absence of cytosol (cytosol –), average pixel intensities obtained for unstressed cells were defined as 100 %. When cytosol was added to semi-intact cells, the combination unstressed cells/unstressed cytosol served as a 100 % control. Means, SDs and statistical significance are shown with unstressed cells or unstressed cells/unstressed cytosol as reference (*, **). In addition, the nuclear accumulation of importin-α and CAS was significantly increased when heat-shocked cells/heat-shocked cytosol was compared with heat-shocked cells/unstressed cytosol (xx). All data points show results from at least three independent experiments; between 54 and 59 cells were quantified in each of these experiments (see Materials and methods for details).

+ NaCl). This is in line with the idea that salt treatment of semi-intact cells released endogenous transport factors from the nuclear envelope, thereby liberating binding sites for fluorescently labeled importin-α and CAS.

Taken together, results in this section are consistent with the idea that the first step of nuclear transport, i.e. docking at the nuclear envelope, is stress sensitive for importin-α and upregulated in response to heat.

Exit of importin-α and CAS from nuclei. The experiments described above indicated that heat shock stimulated significantly the docking of importin-α at the NE, while no drastic changes were observed for CAS. Since nuclear accumulation may result from changes of import, export and/or retention, we tested next whether heat stress affected importin-α or CAS movement from the nucleus to the cytoplasm. To this end, growing cells were heat-shocked and semi-permeabilized with digitonin. During this extraction, a portion of importin-α and CAS was removed from the nucleus (data not shown). Digitonin-treated cells were then incubated in transport buffer supplemented with energy and RanQ69L, conditions that stimulate the formation of export complexes which rely on members of the importin-β family. At different time points during the incubation, intranuclear importin-α and CAS were quantified (Fig. 6B; see Materials and methods for details). Under these conditions, the levels of importin-α decreased somewhat in unstressed cells over time; however, the changes were not statistically significant. In comparison, the levels



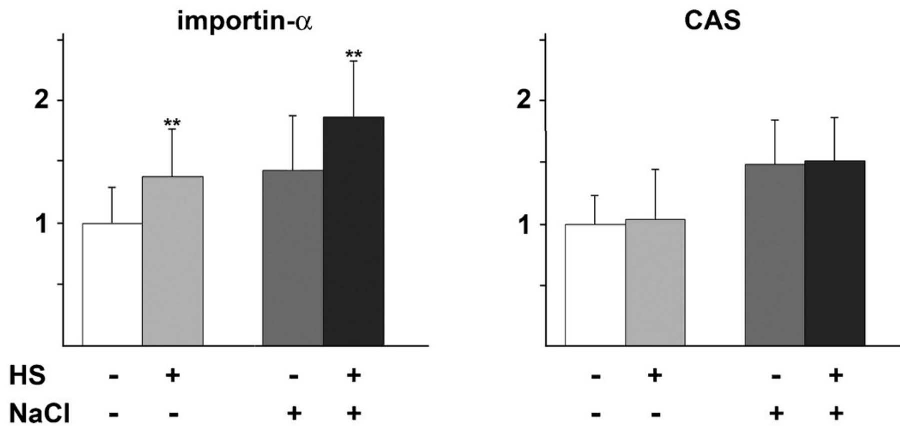


Figure 5. Effect of stress on NE docking of importin-α and CAS. *In vitro* binding of importin-α and CAS was analyzed with semi-intact cells grown at 37 °C or heat-stressed for 30 min at 45.5 °C. Semi-intact cells were pre-treated for 10 min with NaCl before incubation with purified transport factors as indicated. Importin-α or CAS associated with the cytoplasmic side of the NE was visualized by confocal microscopy (*A*), and fluorescence signals associated with NEs were quantified (*B*). Mean NE fluorescence for control cells without salt extraction was defined as 1. Means and SDs are shown; ** $p < 0.01$. Results are representative of at least three independent experiments; between 43–65 cells were quantified for individual data points.

of importin-α remained higher in nuclei of heat-shocked cells at all times; no drastic reduction of nuclear importin-α was observed for stressed cells during the incubation period. Similar results were observed for CAS; although a portion of the carrier moved out of the nucleus in control as well as heat-treated cells, CAS levels in stressed cells remained higher throughout the experiment.

Taken together, these results indicated that upon stress, a larger portion of importin-α did not exit to the cytoplasm; this portion remained concentrated in nuclei during the incubation period. Similarly, elevated levels of CAS remained associated with nuclei of stressed cells. This could be due either to reduced export efficiency, nuclear retention or a combination of these events. Experiments described in the following section analyzed the contribution of retention to the accumulation of importin-α and CAS in nuclei.

Nuclear retention of importin-α and CAS in heat stressed cells. Nuclear retention may interfere with the export of transport factors to the cytoplasm and lead to increased levels of transport factors in the nucleus. We addressed this question by extracting cells with buffer, detergent, DNase, salt, and a mixture of DNase plus RNase (see Materials and methods), using a protocol that was designed to prepare nuclear matrices [26]. For this procedure, all incubations were carried out with unfixed cells. The first step (KM buffer) uses a buffer with low ionic strength and pH 6.2; the shift from growth medium to KM buffer during the first extraction step likely causes the diffuse nuclear, rather than predominantly NE, staining of importin-α in control cells (compare controls in Fig. 7A with Fig. 3A). For unstressed cells, importin-α was efficiently solubilized following treatment with NP40 and DNase incubation. Following DNase and RNase treatment importin-α was almost completely

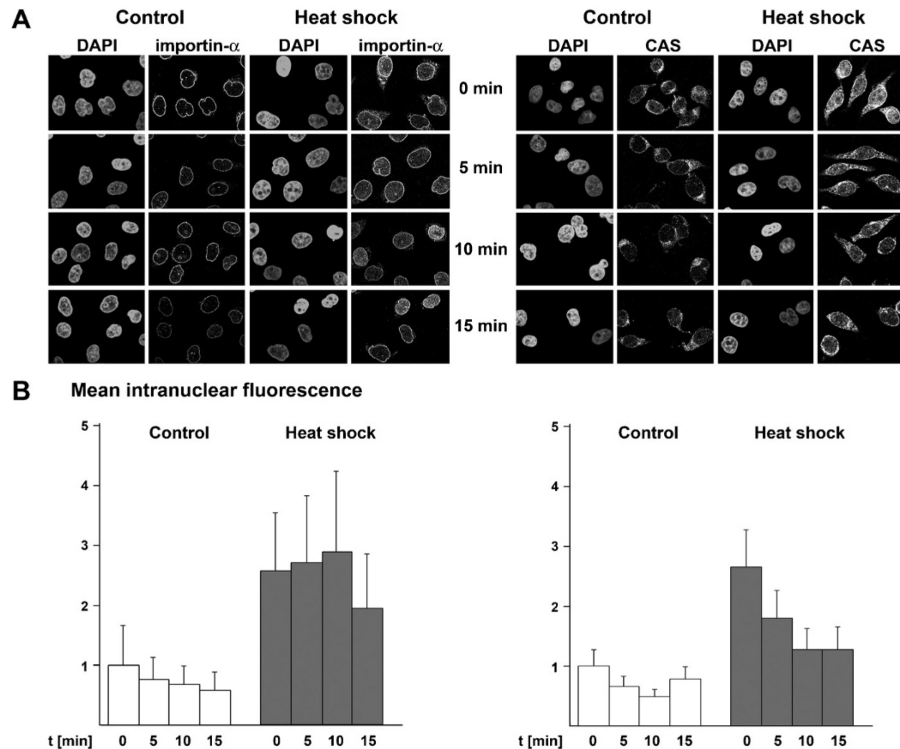


Figure 6. Exit of importin-α and CAS from nuclei of unstressed and heat-shocked cells. Control and heat-shocked HeLa cells were semi-permeabilized with digitonin, and incubated for 5, 10 and 15 min as described in Materials and methods. Cells were fixed and transport factors were detected by indirect immunofluorescence (*A*). Controls (0 min) were fixed immediately after incubation with digitonin. (*B*) The mean (± SD) intranuclear fluorescence was determined for different time points. The mean intranuclear fluorescence of unstressed cells measured at 0 min was defined as 1. Between 48 and 75 cells were quantified for each data point.

extracted from control cells. (Note that all images for Fig. 7A were taken at identical settings.) By contrast, in heat-shocked cells, only a portion of importin-α was solubilized by the different treatments, and high levels of the transport factor remained bound to nuclear structures even after the final extraction step. These results demonstrate that in response to heat shock, a portion of importin-α becomes resistant to extraction. Unlike importin-α, CAS was solubilized efficiently with NP40 in control and stressed cells (Fig. 7B). To detect changes during subsequent steps of the extraction, the detector gain of the confocal microscope was increased (Fig. 7B, part *b*), and all images in part (*b*) were acquired with identical settings. In heat-shocked cells, CAS was somewhat more resistant to the extraction with salt and DNase + RNase when compared to unstressed cells.

In summary, anchorage in nuclei increased in stressed cells for both importin-α and CAS, but the effect was much more prominent for importin-α.

Discussion

The results described here and in previous publications demonstrate that stress inhibits classical nuclear import [16, 17, 20]. This inhibition may be caused by different stress-induced changes of the transport apparatus, and we focused here on the heat-dependent relocation of nuclear transport factors. Importin-α and CAS are key components required for classical protein import; in response to heat shock, the steady-state concentration of both proteins increases in the nuclei of growing cells. We have used *in vitro* transport assays to analyze individual steps of importin-α and CAS nuclear trafficking and to identify the changes triggered by heat shock for each of these reactions. This approach was employed to determine the possible role of cytosolic factors in nuclear import, examine docking at the NE and exit from the nuclear interior. Taken together, our analyses support the conclusion that heat shock affects several of the processes that control the nucleocytoplasmic distribution of importin-α and CAS (summarized in Fig. 8 and Table 1).

In growing cells, heat shock concentrates importin-α and CAS in nuclei, a process determined by nuclear import, retention in the nucleus or cytoplasm and exit from the nucleus. At present, it is not known whether importin-α and CAS in heat-treated cells enter the nucleus by cytosol-independent and/or -dependent routes. Nevertheless, heat upregulates *in vitro* nuclear accumulation via either pathway for CAS and increases cytosol-independent nuclear import for importin-α. Moreover, the combination of cytosol and semi-intact cells indicates that soluble factors from stressed cells may stimulate the nuclear accumulation of importin-α and CAS upon heat shock. The effect of heat stress on cytosol-independent nuclear import

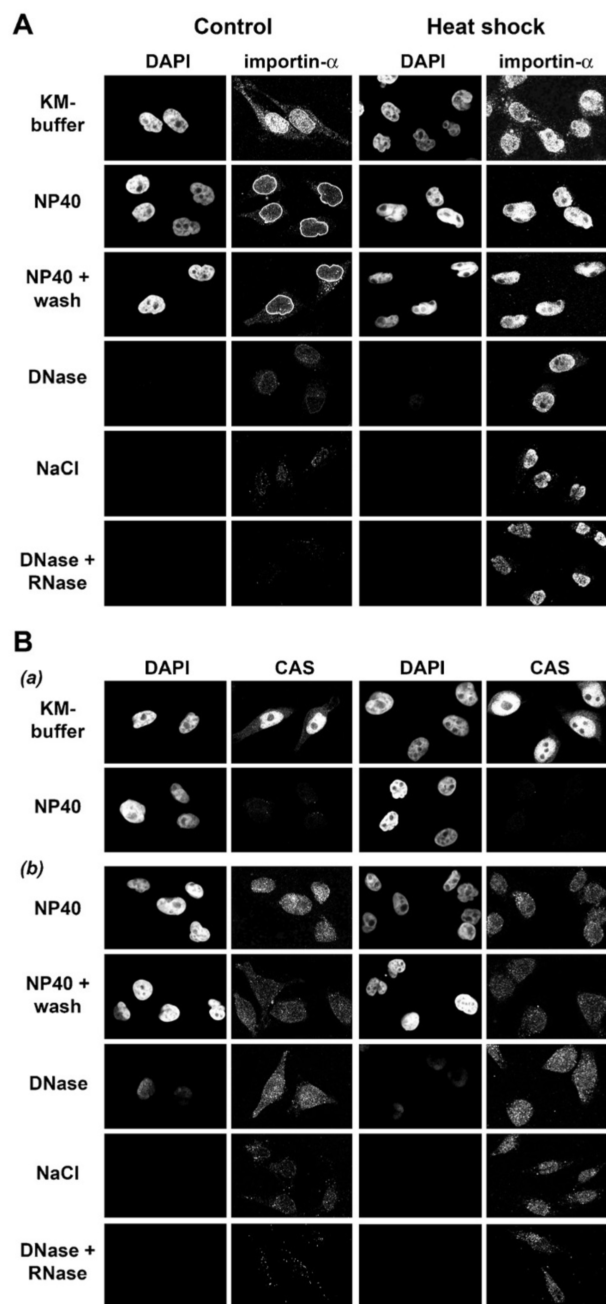


Figure 7. Solubilization of importin-α and CAS in control and heat-shocked cells. Unfixed control and stressed HeLa cells were treated sequentially with KM buffer, the detergent NP40, washed after detergent extraction (NP40 + wash), incubated with DNase, NaCl and a mixture of DNase + RNase. Samples were fixed immediately after each step and processed for indirect immunofluorescence. (*A*) Importin-α which was resistant to extraction by the different treatments, is shown. All images were collected with identical settings. (*B*) Most of CAS was readily extracted with NP40 (a). To monitor potential differences between control and stressed cells following the treatment with NP40, the detector gain was increased and kept constant for all subsequent extraction steps (b). Results shown are representative of at least three independent experiments.

Table 1. Heat alters the nucleocytoplasmic distribution of importin-α and CAS.

	Importin-α	CAS
Steady-state distribution in growing cells	↑ in nuclei	↑ in nuclei
In vitro nuclear accumulation without cytosol	↑	↑
In vitro nuclear accumulation with cytosol	↔	↑
NE docking	↑	↔
In vitro exit from nuclei	↓	↓
Nuclear retention	↑↑↑	(↑)

↑, heat-dependent increase; (↑), minor increase of the process; ↓, stress-induced reduction; ↔, no drastic change in response to stress.

suggests that stress modulates the function of semi-intact cells, possibly by altering components of the NE. As discussed below, for importin-α these stress-induced changes lead to the upregulation of docking. Nuclear import is initiated by docking at the NE, and we show for the first time that heat significantly upregulates the binding of importin-α to the NE, whereas docking of CAS is not drastically altered. Comparable results were obtained with salt-stripped nuclei, suggesting that the free and potential importin-α-binding sites increase upon stress. Based on these results, we propose that heat alters NE components that are involved in docking of importin-α, and nucleoporins are the most likely candidates for these heat-induced changes.

We and others demonstrated previously that stress may affect the retention of transport factors in nuclei [16, 17]. We have extended these studies and show now that heat shock increases nuclear retention of importin-α, which in part becomes resistant to the extraction with detergent, salt and nucleases, indicating drastic changes in solubility. In control cells, importin-α is released following treatment with both NP40 and DNase. Surprisingly, incubation with DNase efficiently liberated importin-α from nuclear envelopes, although only ~4% of the total cellular protein is released with DNase [26]. This may suggest a possible association of importin-α with chromatin at the nuclear periphery in control cells.

In contrast to unstressed cells, heat shock induced a portion of importin-α to become insoluble throughout the fractionation procedure. Although some CAS remains insoluble in stressed cells under these conditions, the effect is much less pronounced and CAS is solubilized to a large extent by detergent in control and heat-treated cells. Taken together, this heat-induced resistance to different extraction steps may suggest a stress-induced association of importin-α

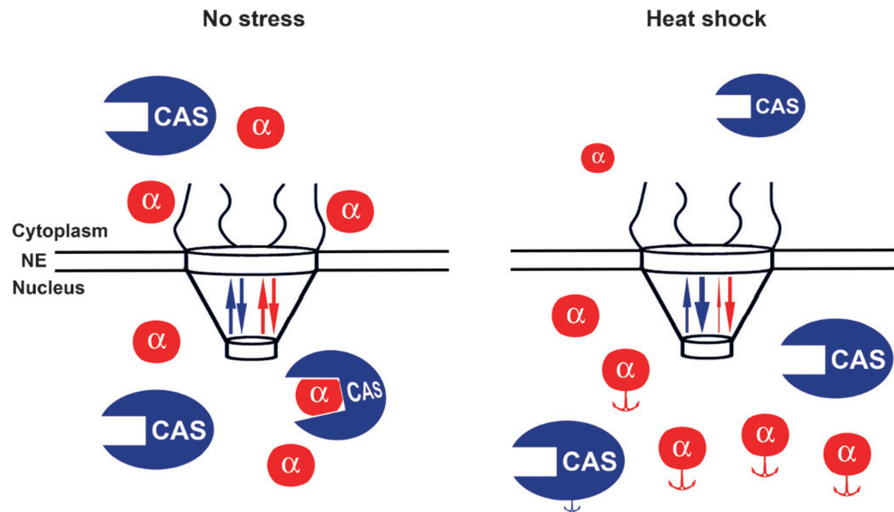


Figure 8. A combination of different mechanisms redistributes importin-α and CAS in heat-shocked cells. In response to heat stress, multiple steps contribute to the nuclear accumulation of importin-α and CAS. These include changes in nuclear import and exit and anchorage in the nucleoplasm. For simplicity, Ran has been omitted from the model. See text for details.

and, to a lesser extent, of CAS with components of the nuclear matrix.

In addition to the analyses of import, docking and retention, our studies address for the first time the exit of importin-α and CAS from nuclei. A cell-free system was used to quantify the levels of transport factors which remain in nuclei under conditions that promote export. These experiments reveal that a fraction of importin-α and CAS does not exit the nucleus in control or stressed cells. However, the levels of transport factors that remain in nuclei are increased by stress, and this effect was possibly somewhat more pronounced for importin-α. It is conceivable that the failure of importin-α to leave the nucleus is a consequence of its anchorage in the nucleoplasm. Similarly, nuclear retention of a small pool of CAS may explain why higher levels of the carrier persist in stressed nuclei, even under conditions that support exit to the cytoplasm. At present, we cannot rule out the possibility that following nuclear exit *in vitro*, a portion of importin-α or CAS is re-imported into nuclei of heat-shocked cells. This re-import could help sustain elevated intranuclear levels of the transport factors.

Based on our results for the exit of importin-α and CAS from nuclei, one might propose that importin-α becomes sequestered in the nucleoplasm by binding to immobilized CAS. However, this seems unlikely, since most of CAS was readily solubilized in stressed cells, whereas importin-α was resistant to extraction. We therefore favor the model that importin-α is anchored in nuclei by associating with nuclear factors other than CAS, and the factors could be components of the nuclear matrix.

Our results support an intricate model for the intracellular distribution of importin-α and CAS, with stress affecting multiple steps (Fig. 8, Table 1). These steps include changes in docking at the NE, nuclear import, the subsequent exit to the cytoplasm and retention in the nuclear interior. Importantly, the impact of heat is different for individual steps of importin-α and CAS nuclear trafficking. Stress-induced changes in NE docking, nuclear exit and retention are of particular importance to concentrate importin-α in nuclei, whereas upregulated nuclear import seems to play a more prominent role for CAS. On the basis of these data, a complex picture emerges for the effect of stress on the distribution of transport factors, nuclear function and organization. Besides changing classical import, it is tempting to speculate that the redistribution of CAS and importin-α will have additional consequences. For example, CAS was shown recently to control the expression of specific genes that are regulated by p53, and importin-α2 interacts with the promoter of the proapoptotic gene *PIG3* [10]. These results support the hypothesis that stress-induced changes in transport factor localization may lead to altered gene expression, thereby regulating the stress response and ultimately cell survival.

In addition to the mechanisms defined with our studies, other processes may play a role in regulating classical nuclear import during aging and for specific forms of neurodegenerative diseases. Thus, an age-dependent decline in import efficiency correlates with decreasing levels of transport factors in old human fibroblasts [6], changes we did not detect in heat-stressed cells. Interestingly, a mislocalization of importin-α1 was observed in hippocampal neurons from Alzheimer patients [27]. In these cells, the transport factor accumulates in Hirano bodies, inclusions located in the cytoplasm, which may affect nuclear trafficking [27]. Likewise, in a mouse model of amyotrophic lateral sclerosis, importin-α distribution was shifted toward the cytoplasm with reduced levels

in the nucleus [28]. Together with our results, these data suggest that several pathophysiological states, as exemplified by neurodegenerative diseases or stress, cause a relocation of transport factors. This unbalanced distribution of essential transport components is likely to impair nuclear trafficking, thereby interfering with correct nucleocytoplasmic communication and other cellular functions.

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