

Cellular stresses induce the nuclear accumulation of importin α and cause a conventional nuclear import block

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We report here that importin α accumulates reversibly in the nucleus in response to cellular stresses including UV irradiation, oxidative stress, and heat shock. The nuclear accumulation of importin α appears to be triggered by a collapse in the Ran gradient, resulting in the suppression of the nuclear export of importin α . In addition, nuclear retention and the importin β /Ran-independent import of importin α also facilitate its rapid nuclear accumulation. The findings herein show that the

classical nuclear import pathway is down-regulated via the removal of importin α from the cytoplasm in response to stress. Moreover, whereas the nuclear accumulation of heat shock cognate 70 is more sensitive to heat shock than the other stresses, importin α is able to accumulate in the nucleus at all the stress conditions tested. These findings suggest that the stress-induced nuclear accumulation of importin α can be involved in a common physiological response to various stress conditions.

Introduction

In the cytoplasm, a cargo containing a classical NLS that consists of a short stretch of basic amino acids is recognized by importin (karyopherin) α and forms a stable ternary complex with importin (karyopherin) β . The complex interacts with the nuclear pore complex, which is mediated by importin β and is translocated into the nucleoplasm. In the nucleus, a Ras-related small nuclear GTPase, Ran, is localized mainly as the GTP-bound form, and the nuclear RanGTP causes the dissociation of the complex by binding to importin β . These transport factors are recycled back to the cytoplasm to transport the next karyophile into the nucleus (Adam and Gerace, 1991; Görlich and Mattaj, 1996; Jans et al., 2000). In this fashion, Ran appears to be important for the loading and unloading of cargoes on transport receptors, and thus plays a key role in determining the directionality of nuclear transport (Yoneda et al., 1999).

A variety of cellular stresses affect multiple aspects of cellular physiology, and an important part of the cellular stress response involves the translocation of stress-responsible factors from the cytoplasm to the nucleus. However, how these factors are translocated into the nucleus in response to stress or how the nuclear transport pathways are regulated in stressed cells is unclear.

In this paper, we report that importin α accumulates in the nucleus in response to cellular stresses including UV irradiation, oxidative stress, and heat shock stress, resulting in the inhibition of classical nuclear import. We demonstrated that the decrease in nuclear RanGTP levels actually triggers the accumulation of importin α in the nucleus by suppressing nuclear export. Moreover, we show that both nuclear retention and the importin β /Ran-independent nuclear import of importin α are also involved in its rapid nuclear accumulation.

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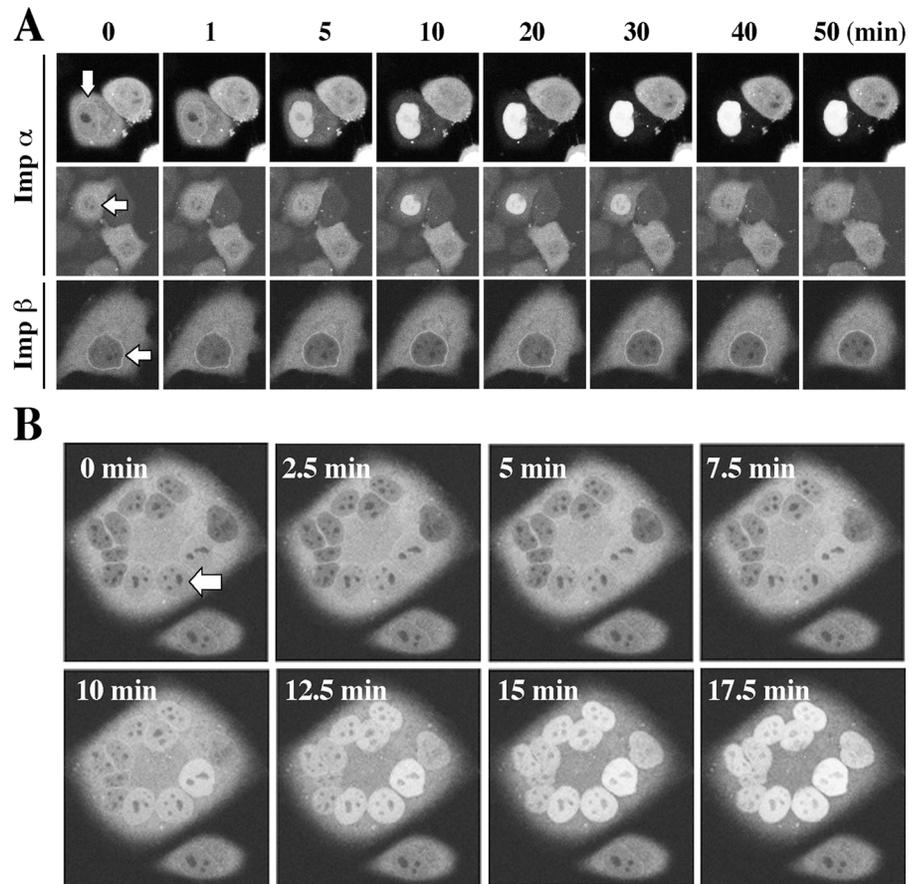
Results and discussion

We attempted to examine the dynamic behavior of importin α under a variety of physiological cell conditions using a live cell imaging technique. In this work, we first investigated

Abbreviation used in this paper: hsc, heat shock cognate.

Figure 1. Importin α accumulates in the nucleus in response to UV irradiation.

The nuclei of cells expressing either EGFP-importin α or β were irradiated with UV (364 nm) using a laser in conjunction with the confocal microscopy, and the subcellular localization of EGFP fusion proteins was observed by time-lapse photography. (A, top and middle) EGFP-importin α -expressing cells. (bottom) EGFP-importin β -expressing cell. The nucleus (arrows) was irradiated with UV. (B) After EGFP-importin α -expressing cells were fused by hemagglutinating virus of Japan, one nucleus in the fused cell (arrow) was irradiated with UV and the cell was scanned.



the behavior of importin α in UV-irradiated cultured cells. HeLa cells were transiently transfected with the pEGFP-importin α plasmid. After incubation for 24 h at 37°C, the cells were stained with Hoechst 33342 and the cell nucleus was irradiated with a UV laser (364 nm) using a confocal laser-scanning microscope. Upon UV irradiation of the nucleus (Fig. 1 A, arrows), EGFP-importin α was observed to rapidly accumulate in the nucleus (Fig. 1 A, top and middle; and Video 1 and 2, available at <http://www.jcb.org/cgi/content/full/jcb.200312008/DC1>). When only the cytoplasm was irradiated using the same procedure, no nuclear accumulation of EGFP-importin α was observed (unpublished data). In contrast to importin α , EGFP-importin β showed no change in distribution (Fig. 1 A, bottom; and Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200312008/DC1>). In addition, we observed that importin α accumulated in the nucleus of UV-irradiated cells in two distinct manners, irreversibly (Fig. 1 A, top) and reversibly (Fig. 1 A, middle). Although we cannot precisely explain the molecular mechanism for this difference, it is likely that the difference in reversibility is due to experimentally uncontrollable variations in the UV dose used.

To confirm cell viability, we irradiated the same nucleus a second time, after EGFP-importin α redistributed to the cytoplasm. EGFP-importin α again accumulated in the nucleus in a similar manner (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200312008/DC1>). These results indicate that cells in which EGFP-importin α shut-

tles between the cytoplasm and the nucleus in response to the UV irradiation are viable.

To determine if the nuclear accumulation of importin α in response to UV irradiation is directly involved in the repair of UV-damaged DNA, we examined the issue of whether importin α effectively accumulates only in the irradiated nucleus but not other nonirradiated nuclei of a polykaryon constructed by cell fusion. At 30 min after the fusion of HeLa cells transfected with pEGFP-importin α , only one nucleus (Fig. 1 B, arrow) was irradiated by UV. As shown in Fig. 1 B (Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200312008/DC1>), EGFP-importin α accumulated in all the nuclei of the fused cells at almost the same rate. In addition, the nuclear accumulation of EGFP-importin α was also reversible in the polykaryon (unpublished data). Therefore, it is likely that the nuclear accumulation of importin α induced by UV irradiation is the result of cellular responses to UV irradiation, probably one of the stress responses, rather than that of the repair of damaged DNA.

Next, to determine whether or not the nuclear accumulation of importin α is a common response to various stress conditions, we treated HeLa cells with hydrogen peroxide (H_2O_2) or incubated them at 42°C. When the cells were incubated in a medium including 200 μM H_2O_2 at 37°C, endogenous importin α detected by antibodies specific for importin α was rapidly localized in the nucleus after approximately a 30-min incubation (Fig. 2 A). Furthermore, when the cells were placed in fresh medium without H_2O_2 after

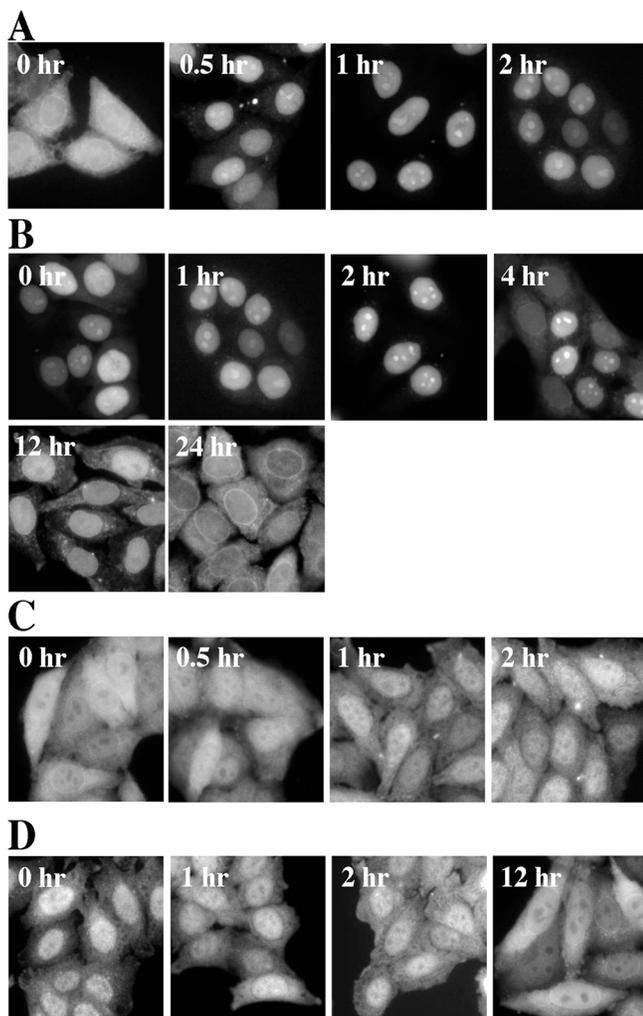


Figure 2. Importin α accumulates in the nucleus in response to either the oxidative stress or the heat shock stress. After HeLa cells were treated with hydrogen peroxide or incubated at 42°C at the indicated time, the cells were stained by specific antibodies for importin α (mRch1) and Alexa488-conjugated secondary antibodies. (A) HeLa cells were cultured with a medium containing 200 μ M H₂O₂. (B) After HeLa cells were treated with 200 μ M H₂O₂ for 1 h, the cells were incubated with fresh medium in the absence of H₂O₂. (C) HeLa cells were incubated at 42°C. (D) After HeLa cells were incubated for 1 h at 42°C, they were incubated at 37°C for the indicated times.

treatment with H₂O₂ for 1 h, the importin α began to redistribute to the cytoplasm within 4 h, and the distribution of endogenous importin α returned to the original state 24 h after the replacement (Fig. 2 B).

In a subsequent experiment, HeLa cells were incubated at 42°C. The endogenous importin α showed a significant nuclear distribution at 1 h after heat shock (Fig. 2 C), although the extent of the nuclear accumulation induced by heat shock was apparently low compared with oxidative stress. Similar to oxidative stress, when the cells were returned to 37°C after a heat shock treatment for 1 h, the subcellular localization of endogenous importin α returned to the normal cytoplasmic distribution after 12-h incubation at 37°C (Fig. 2 D).

Based on the data showing that importin α accumulates in the nucleus in response to cellular stress, we hypothesized

that the nuclear import efficiency of the classical NLS substrates is decreased due to the suppressed recycling of importin α to the cytoplasm. To analyze the import rate, we performed time-lapse experiments. After treating HeLa cells with H₂O₂ for 30 min, recombinant GST-SV40 T antigen NLS-GFP (GST-NLS-GFP) proteins were injected into the cytoplasm, and the rate of nuclear import of the substrate was measured under the stress conditions. In unstressed cells, the substrate rapidly migrated into the nuclei (Fig. 3 A, top). In contrast, in stress-exposed cells the nuclear import rate of the substrate was dramatically diminished, although eventually substrates gradually accumulated in the nucleus (Fig. 3 A, bottom).

To address the issue of whether or not the suppression of nuclear import rate of the NLS substrate is primarily due to the cytoplasmic depletion of importin α , recombinant importin α proteins were coinjected with the substrate into the cytoplasm of H₂O₂-exposed cells. As shown in Fig. 3 B (top), the import rate of the NLS substrate was restored significantly but not completely even under stress conditions by the recombinant importin α . In contrast, the recombinant importin β had no effect on import efficiency (Fig. 3 B, bottom). Furthermore, the same phenomenon was observed under heat shock conditions (unpublished data). Collectively, these results indicate that the import defect of classical NLS-containing karyophiles under stress conditions is primarily caused by the depletion of importin α from the cytoplasm, although we cannot exclude the possibility completely that the nuclear accumulation of importin α is not directly relevant to the defect of classical import pathway.

What induces the nuclear accumulation of importin α ? It is possible that the stress-induced nuclear accumulation of importin α is caused by the inhibition of its export pathway. Because it is known that the cellular apoptosis susceptibility gene product CAS (also referred to as exportin 2) exports importin α from the nucleus in a nuclear RanGTP-dependent manner (Kutay et al., 1997), we analyzed the distribution of CAS under the stress conditions using indirect immunofluorescence. Endogenous CAS showed no change in distribution under any of the stress conditions tested (unpublished data). Next, we attempted to examine if the subcellular localization of Ran is altered in response to cellular stress. HeLa cells were incubated with H₂O₂ for 1 h, irradiated with UV, or incubated at 42°C for 1 h, and the subcellular localization of endogenous Ran was observed using indirect immunofluorescence. As shown in Fig. 4 A, a significant amount of Ran was distributed to the cytoplasm in response to all three types of stress. In addition, in each stress condition, the level of the cytoplasmic distribution of Ran was mutually correlated with that of the nuclear accumulation of importin α , suggesting that the stress-induced alteration in Ran distribution may suppress the efficiency of the CAS-mediated nuclear export of importin α .

To address the issue of whether or not the decline in nuclear RanGTP is actually related to the nuclear accumulation of importin α , recombinant GST-fused GTPase-deficient mutant of Ran, GST-Q69LRanGTP proteins were injected into the nucleus of HeLa cells to supply the nuclear RanGTP, and the injected cells were exposed to H₂O₂ for 30 min. As shown in Fig. 4 B (top) endogenous importin α

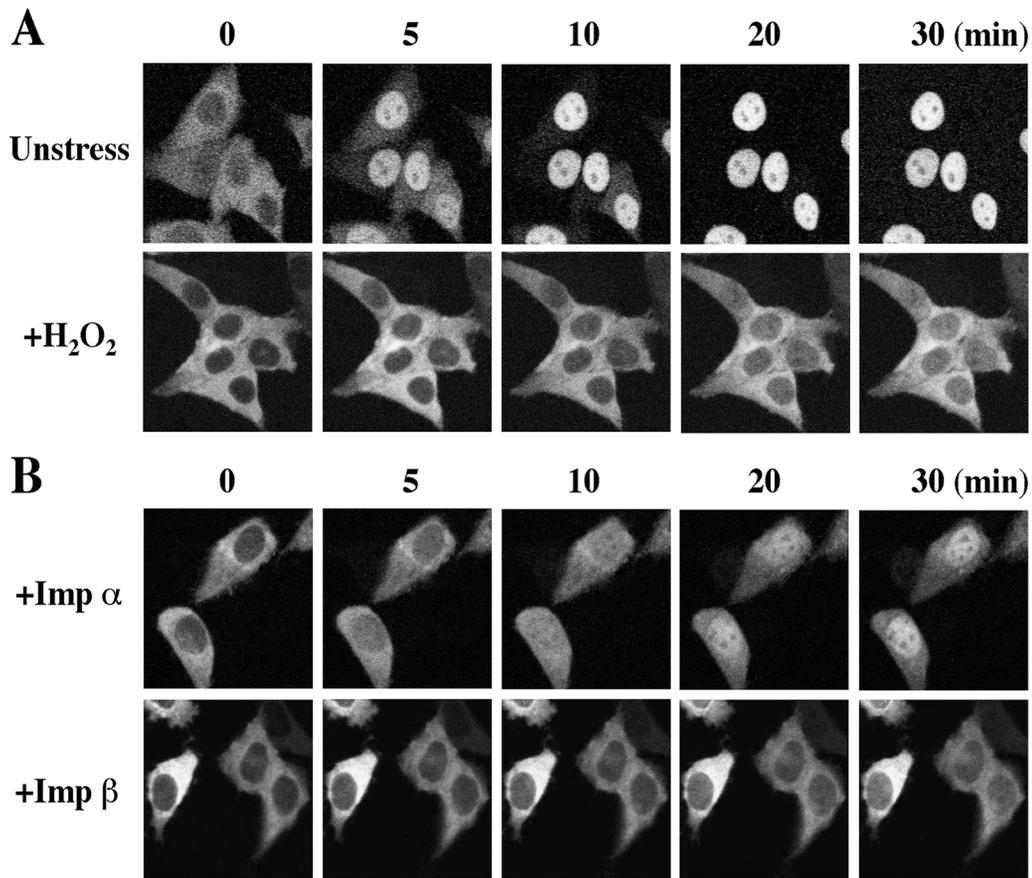


Figure 3. The classical nuclear import pathway is suppressed under conditions of stress. After HeLa cells were treated without or with 200 μ M H₂O₂ for 30 min, 1 mg/ml GST-NLS-GFP was injected into the cytoplasm of the cells without (A) or with (B) a molar equivalent of recombinant importin α or β , and the subcellular localization of GST-NLS-GFP was observed by time-lapse photography. Images were collected at 10-s intervals.

was found to be localized exclusively in the cytoplasm of the injected cells under the oxidative stress conditions, whereas in noninjected cells, importin α rapidly accumulated in the nucleus. To exclude the possibility that the nuclear injection of Q69LRanGTP blocked the import of importin α , GFP-importin α was injected into the cytoplasm of the same cells under nonstress conditions after the nuclear injection of GST-Q69LRanGTP. We found that the nuclear injection of Q69LRanGTP did not suppress the import of importin α (unpublished data). These results indicate that the collapse of Ran distribution leading to the suppression of the CAS-mediated nuclear export of importin α plays a pivotal role in the stress-induced nuclear accumulation of importin α .

In contrast, after pretreating the HeLa cells with H₂O₂ for 30 min, GST-Q69LRanGTP was injected into the nucleus and the cells were incubated for 30 min under the stress condition. Indirect immunofluorescence showed that endogenous importin α was detected in the nucleus (Fig. 4 B, bottom), suggesting that importin α can be retained in the nucleus after migration induced by the collapse of the Ran gradient. Consistently, we observed that DNase I treatment, but not RNase, dramatically abolished nuclear importin α localization (unpublished data). It should be noted that a variety of NLSs overlap with DNA-binding regions (Cokol et al., 2000). Therefore, it is possible that importin α migrates

into the nucleus to bind to the NLS regions of DNase I-sensitive chromatin components exposed as the result of the stress conditions. It is reasonable to speculate that the nuclear retention of importin α after nuclear migration in stress-exposed cells may accelerate the rapid nuclear accumulation of importin α triggered by a collapse of the Ran gradient.

Consistent with our results, it has recently been reported that alterations in Ran distribution play a role in regulating nucleocytoplasmic transport under stress conditions (Czubryt et al., 2000; Stochaj et al., 2000). What induces the distribution change of Ran? Czubryt et al. (2000) reported that activation of the mitogen-activated protein kinase ERK2, an extracellular signal-regulated kinase, mediates the collapse of the Ran gradient by H₂O₂ treatment. In addition, it should be noted that nuclear import efficiency is affected by the activation of kinases (Kehlenbach and Gerace, 2000). Thus, it will be intriguing to determine whether kinase/phosphatase or other stress-inducible modifications directly alter the distribution of Ran under different stress conditions.

In contrast, one can speculate that nucleotide depletion may affect the Ran gradient. It has been shown that DNA strand breaks result in a drop in cellular ATP levels by the consumption of NAD for poly(ADP-ribose) synthesis (Carson et al., 1986). In addition, UV irradiation was shown to

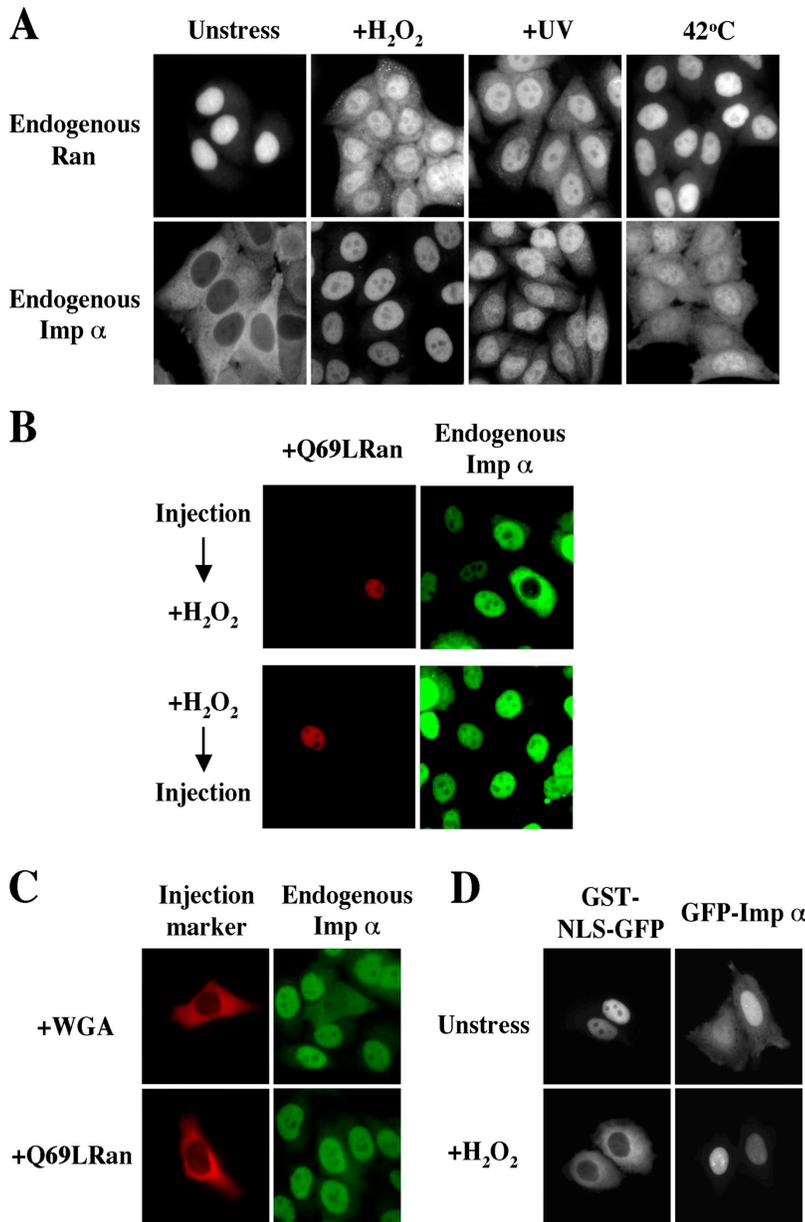


Figure 4. Distribution change of Ran in response to the cellular stresses induces the nuclear accumulation of importin α . (A–C) After HeLa cells were exposed to various stresses, indirect immunofluorescence was performed to detect either endogenous importin α (mRch1) or endogenous Ran using specific antibodies. (B and C) Green, importin α stained with Alexa488-conjugated second antibodies; red, an injection marker (Alexa568-conjugated antibodies). (A) HeLa cells were incubated with 200 μ M H₂O₂ for 1 h, irradiated with UV (254 nm, 0.3 J/cm²), or incubated at 42°C for 1 h. (B, top) After 4 mg/ml GST-Q69LRanGTP was injected into the nucleus of HeLa cells, they were further incubated with 200 μ M H₂O₂ for 30 min. (bottom) After HeLa cells were treated with 200 μ M H₂O₂ for 30 min, 4 mg/ml GST-Q69LRanGTP was injected into the nucleus of HeLa cells, and they were further incubated for 30 min. (C) After either 2 mg/ml WGA or 8 mg/ml GST-Q69LRanGTP was injected into the cytoplasm of HeLa cells, they were further incubated with 200 μ M H₂O₂ for 30 min. (D) After HeLa cells were incubated with 200 μ M H₂O₂ for 30 min, either 1 mg/ml of GST-NLS-GFP or GFP-importin α was injected into the cytoplasm of the cells, and then they were further incubated for 30 min.

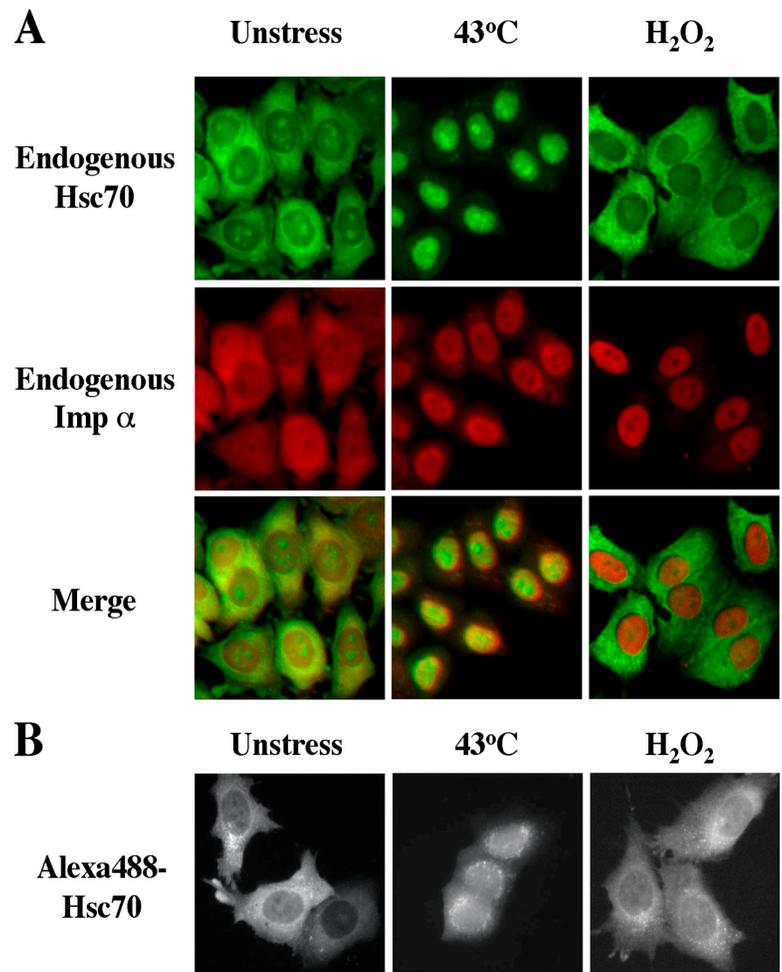
directly induce a decrease in mitochondrial respiratory activity, leading to ATP depletion (Djavaheri-Mergny et al., 2001). It has also been reported that hydrogen peroxide treatment results in lowered cellular ATP levels (Wu et al., 1996). In fact, Schwoebel et al. (2002) demonstrated that when cells were depleted of ATP by the addition of sodium azide and 2-deoxyglucose, Ran-dependent nuclear transport was rapidly inhibited. Thus, the drop in ATP level can lead to intracellular GTP depletion by the cellular interconversion of nucleotides. Therefore, a drop in GTP concentration should affect the production of RanGTP in the nucleus, resulting in the collapse of the Ran gradient. Further work will be required to address what actually triggers the change in distribution of Ran.

Next, we analyzed the nuclear import manner of importin α in response to the cellular stress. It has already been shown that importin α is able to migrate into the nucleus via two independent pathways, importin β /Ran-dependent and

-independent (Miyamoto et al., 2002). It is known that, whereas WGA inhibits both pathways, Q69LRanGTP blocks only the importin β /Ran-dependent pathway. The nuclear import of the injected GST-NLS-GFP was consistently blocked by the coinjection of either WGA or GST-Q69LRanGTP, whereas that of the injected GFP-importin α was not inhibited by the coinjection of GST-Q69LRanGTP (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200312008/DC1>).

Under the same assay conditions, WGA or GST-Q69LRanGTP was injected into the cytoplasm of HeLa cells, and the cells were incubated in the presence of H₂O₂ for 30 min. As shown in Fig. 4 C, endogenous importin α did not accumulate in the nucleus of the WGA-injected cells, whereas the importin α became localized in the nucleus of the Q69LRanGTP-injected cells, like the surrounding uninjected cells. Furthermore, after HeLa cells were exposed to H₂O₂ for 30 min, GFP-importin α was injected into the cy-

Figure 5. The nuclear accumulation of importin α , but not hsc70, is commonly observed in response to various stresses. (A) HeLa cells were either exposed to 43°C for 1 h or incubated with 200 μ M H₂O₂ for 1 h. The cells were stained by either endogenous importin α (mRch1) or endogenous hsc70 using specific antibodies. Green, hsc70, Alexa488-conjugated second antibodies; red, importin α , Alexa568-conjugated second antibodies. (B) After HeLa cells were either exposed to 43°C for 30 min or incubated with 200 μ M H₂O₂ for 30 min, Alexa488-labeled recombinant hsc70 proteins were injected into the cytoplasm of the cells, and they were incubated for 1 h at 43°C or in the presence of 200 μ M H₂O₂.



toplasm of the cells followed by incubation for 30 min. Under the condition, in which GST-NLS-GFP was not transported into the nucleus, a considerable amount of GFP-importin α accumulated in the nucleus (Fig. 4 D). These results indicate that, in stress-induced cells, importin α is able to migrate into the nucleus, at least in part, in an importin β /Ran-independent manner. Along with the other aforementioned findings, we propose that the nuclear accumulation of importin α in response to cellular stress can occur through a combination of the following events: (a) the inhibition of the nuclear export of importin α by CAS, resulting from a collapse in Ran distribution, (b) the nuclear retention of importin α , and (c) the importin β /Ran-independent nuclear import of importin α .

We demonstrated that the nuclear accumulation of importin α in response to the cellular stresses actually suppressed the classical NLS nuclear import pathway *in vivo* (Fig. 3). To address the issue of how the nuclear transport pathway for classical NLS-containing proteins and that of stress-responsible factors are mutually regulated in stressed cells, we focused on heat shock cognate (hsc) 70, a well known typical heat shock protein that appears to be able to enter the nucleus efficiently without the aid of the classical nuclear import pathway under conditions of stress (Lamian et al., 1996).

We first confirmed the subcellular localization of hsc70 under the stress conditions using a specific antibody. HeLa

cells were either exposed to 43°C for 1 h or incubated with 200 μ M H₂O₂ for 1 h. In this case, to observe the nuclear localization of hsc70 clearly, the cells were incubated at 43°C instead of 42°C. As expected, endogenous hsc70 accumulated in the nucleus under heat shock condition (Fig. 5 A). In contrast, under conditions of oxidative stress, hsc70 was mainly localized in the cytoplasm and was slightly detected in the nucleoli, although importin α clearly accumulated in the nucleus (Fig. 5 A). In UV-exposed cells, hsc70 was also observed mainly in the cytoplasm, analogous to oxidative stress (unpublished data).

After the cells were exposed to the same stresses as described in Fig. 5 A, Alexa488-labeled hsc70 proteins were injected into the cytoplasm of the cells followed by incubation for 1 h under stress conditions. As shown in Fig. 5 B, the hsc70 migrated into the nucleus under heat shock conditions but not under oxidative stress. These results indicate that the nuclear accumulation of hsc70 is much more sensitive to heat shock than oxidative stress, which is consistent with a previous paper (Chu et al., 2001). That is, the nuclear accumulation of hsc70 occurs in a heat stress-specific manner, and hsc70 may play some specific roles in the nucleus to restore the heat-induced damage of proteins.

In contrast, importin α clearly accumulated in the nucleus under all stress conditions tested, suggesting that importin α may accumulate and function in the nucleus as one of the

common responses induced by a variety of stresses. It would be interesting to know if importin α is able to play a role in the intranuclear response specific for stresses as a “nuclear stress response.”

Materials and methods

Cell culture

HeLa cells were incubated in Dulbecco's modified MEM (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS at 37°C in a 5% CO₂ atmosphere.

Transfection and UV irradiation

Importin α (mouse Rch1) and β were constructed with pEGFP-C1 plasmid (CLONTECH Laboratories, Inc.). HeLa cells were plated onto a glass bottom dish (MatTek Corporation) cultured for 1–2 d before use. After the constructs were transfected using Effecten Transfection Reagent (QIAGEN) under the conditions recommended by the supplier for 24 h, the cells were treated with 1 μ g/ml of Hoechst 33342 in culture medium without phenol red, followed by incubation for 15 min at 37°C. The cells were placed on the stage of a confocal laser-scanning microscope (model LSM510; Carl Zeiss MicroImaging, Inc.). A nucleus (\sim 100 μ m²) of the cells expressing the EGFP fused proteins was irradiated with a UV (364 nm) laser (iteration 50, a power of 100% transmission of 0.055–0.057 mW was used). Images were collected at 10- or 20-s intervals after irradiation of the nucleus.

As described previously (Miyamoto et al., 2002), when the expression level was high, we observed the nuclear localization of transiently expressed EGFP-importin α even in the absence of stress. However, when the expression level was not so high, transiently expressed EGFP-importin α was found to remain in the cytoplasm. In this work, we selected EGFP-importin α -expressing cells in which the expression level was not so high to assess the stress response more easily and convincingly.

Time-lapse imaging

Cells were plated on a glass bottom dish (MatTek Corporation) and maintained with MEM supplemented with 10% FBS, antibiotics, 8 mM of L-glutamine, and 30 mM Hepes, pH 7.1, without phenol red. Experiments were performed on an inverted microscope (model Axiovert 100M; Carl Zeiss MicroImaging, Inc.) equipped with a stage (model CZI-3; Carl Zeiss MicroImaging, Inc.) heated to 37°C in conjunction with an objective heater (Biopetechs) and a 63 \times NA 1.4 Plan-Apochromat (Fig. 1 and Fig. S1) or 40 \times NA 0.6 LD-Achroplan (Fig. 3). Time-lapse imaging was performed using a confocal laser-scanning microscope equipped with 488-nm lasers in conjunction with a BP 505–550 for EGFP. The acquired images were processed by LSM510 software version 3.2 SP2.

Indirect immunofluorescence

Indirect immunofluorescence was performed as described previously (Miyamoto et al., 2002). The mAbs against importin α (mRch1) and Ran (BD Biosciences) were used at 1:250 and 1:500, respectively. Anti-hsc70 antibodies (StressGen Biotechnologies) were used at 1:100.

Expression and purification of recombinant proteins

Recombinant untagged and GFP fused importin α (mouse Rch1) and β , GST-SV40 T antigen NLS-GFP (GST-NLS-GFP), and GST-Q69LRanGTP were prepared as described previously (Miyamoto et al., 2002). Full-length cDNA of hsc70 subcloned into pGEX6P-1 was provided by S. Kose (RIKEN, Wako, Japan). Purification of the recombinant hsc70 protein was performed in the same manner as for GFP-importin α . GST-free hsc70 was labeled with Alexa Fluor 488 dye (Molecular probes) according to the manufacturer's recommendation.

Cell fusion by Sendai virus

HeLa cells cultured on a glass bottom dish were transfected with pEGFP-importin α and incubated for 24 h. The cells were fused as described previously (Tachibana et al., 1994). Hemagglutinating virus of Japan (Sendai virus) was provided by M. Nakanishi (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan).

Online supplemental material

Video 1 shows an irreversible nuclear accumulation of EGFP-importin α in a UV-irradiated cell. Video 2 shows a reversible nuclear accumulation of

EGFP-importin α in a UV-irradiated cell. Video 3 shows a subcellular localization of EGFP-importin β in a UV-irradiated cell. Video 4 shows an irreversible nuclear accumulation of EGFP-importin α in a UV-irradiated fusion cell. Fig. S1 shows that importin α accumulated in the nucleus repeatedly in response to the UV irradiation. Fig. S2 shows that importin α migrated into the nucleus in an importin β /Ran-independent manner in an in vivo assay. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200312008/DC1>.

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