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Multiple mechanisms promote the inhibition of classical nuclear import upon exposure to severe oxidative stress

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Abstract

In growing HeLa cells, severe stress elicited by the oxidant hydrogen peroxide inhibits classical nuclear import. Oxidant treatment collapses the nucleocytoplasmic Ran concentration gradient, thereby elevating cytoplasmic GTPase levels. The Ran gradient dissipates in response to a stress-induced depletion of RanGTP and a decreased efficiency of Ran nuclear import. In addition, oxidative stress induces a relocation of the nucleoporin Nup153 as well as the nuclear carrier importin-β, and docking of the importin-α/β/cargo complex at the nuclear envelope is reduced. Moreover, Ran, importin-β and Nup153 undergo proteolysis upon oxidative stress. Caspases and the proteasome degrade Ran and importin-β; however, ubiquitination of these transport factors is not observed. Inhibition of caspases in stressed cells alleviates the mislocalization of importin-β, but does not restore the Ran concentration gradient or classical import. In summary, inhibition of classical nuclear import by hydrogen peroxide is caused by a combination of multiple mechanisms that target different components of the transport apparatus.
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Abbreviations: BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; NE, nuclear envelope; NLS, nuclear localization signal; NPC, nuclear pore complex; RBD, Ran-binding domain; TMR, tetramethylrhodamine

Introduction

Stress is implicated in the pathophysiology of ischemia, heart failure, hypertension and cancer. Moreover, the appropriate response to oxidative stress determines aging and influences the life span of an organism (reviewed in Finkel and Holbrook[1]). On a subcellular level, stress changes the nucleocytoplasmic distribution of proteins (reviewed in Hood and Silver[2]). For instance, in cells that have been treated with reactive oxygen species, a variety of transcription factors relocalize within the cell to modulate gene expression (reviewed in Finkel and Holbrook[1] and Allen and Tresini[3]). Therefore, defining the response to stress on a subcellular level, in particular with respect to nucleocytoplasmic trafficking of macromolecules, will contribute significantly to our understanding of the stress response and stress-related pathophysiology.

Oxidative stress activates several signaling cascades, including the MAPK family members ERK1/2, JNK/SAPK and p38. Likewise, the PI(3)-kinase/Akt pathway becomes active (reviewed in Finkel and Holbrook,[1] Robinson and Cobb[4] and Rhee[5]). Importantly, cell-specific differences in the activation of signaling pathways have been observed (reviewed in Robinson and Cobb[4]). In HeLa cells, hydrogen peroxide induces phosphorylation of the MAPKs ERK, JNK/SAPK and p38.[6] Hydrogen peroxide not only activates several signal transduction pathways, it may also activate the nuclear proteasome, thereby increasing the degradation of proteins damaged by oxidants.[7]

Nucleocytoplasmic trafficking of macromolecules is sensitive to stress; heat shock, ethanol and oxidative stress inhibit classical nuclear protein import.[8,9,10] In growing yeast cells and in semi-permeabilized smooth muscle cells *in vitro*, oxidants have been shown to interfere with classical nuclear import. In these model systems, redistribution of the small GTPase Gsp1p/Ran from the nucleus to the cytoplasm is believed to contribute to nuclear import inhibition.[8,9] In digitonin-treated aortic smooth muscle cells, the MAPK ERK2 plays a role in classical transport inhibition triggered by hydrogen peroxide, and transport inhibition can be overcome if ERK2 activation is prevented.[9] So far, the effect of oxidative stress on classical nuclear import in growing mammalian cells has not been studied.

Like cytoplasmic transport factors, nucleoporins are involved in nucleocytoplasmic trafficking of proteins and RNA (reviewed in Stochaj and Rother,[11] Görlich and Kutay[12] and Kuersten *et al.*[13]). For instance, Nup153, a member of the FXFG family of nucleoporins, is located at the nuclear basket, where it participates in the termination of classical nuclear protein import.[14] Despite these previous studies, it is presently not clear how trafficking across the NPC changes in response to stress. To address these questions, we have now analyzed the effect of oxidative stress on classical nuclear import in growing HeLa cells and *in vitro*. Our results show that components of the nuclear transport apparatus are relocated in cells treated with hydrogen peroxide. Furthermore, exposure to severe oxidative stress decreases the availability of RanGTP and induces the degradation of Ran, importin-β and Nup153, components required for classical and several nonclassical nuclear trafficking pathways.

Results and Discussion

Oxidative stress inhibits classical nuclear import in growing HeLa cells

To determine how oxidative stress affects classical nuclear import in growing mammalian cells, we transiently transfected HeLa cells with plasmids encoding either NLS-GFP, a fluorescent reporter protein, which carries SV40-NLS fused to GFP, or the GFP-tag only.[10] The small size of NLS-GFP allows diffusion across the NPC, a process independent of active transport. In addition, the simple SV40-NLS is recognized by the classical transport apparatus, which imports NLS-GFP into the nucleus. The net distribution of the reporter protein is a combination of diffusion and transport, and nuclear accumulation requires classical import to be active. Concentrated in nuclei under normal conditions (Figure 1A, panel b), NLS-GFP also appears in the cytoplasm upon exposure to hydrogen peroxide (Figure 1A, d). By contrast, the localization of GFP, which can be found in the nucleus and cytoplasm, was not altered by this treatment (Figure 1A, f, h), supporting the idea that oxidative stress does not prevent diffusion across NPCs. Therefore, a redistribution of NLS-GFP in response to oxidative stress can be attributed to changes in classical import.

In initial experiments, we have tested the effect of different concentrations of hydrogen peroxide on NLS-GFP localization, and a 1 h treatment with 10 mM hydrogen peroxide was found to be optimal to mislocalize a portion of NLS-GFP to the cytoplasm. At lower concentrations, NLS-GFP remained accumulated in the nuclei. By contrast, upon addition of 20 mM of the oxidant, NLS-GFP completely equilibrated within the cells, and similar fluorescent signals were obtained for the nucleus and cytoplasm (not shown). On the basis of

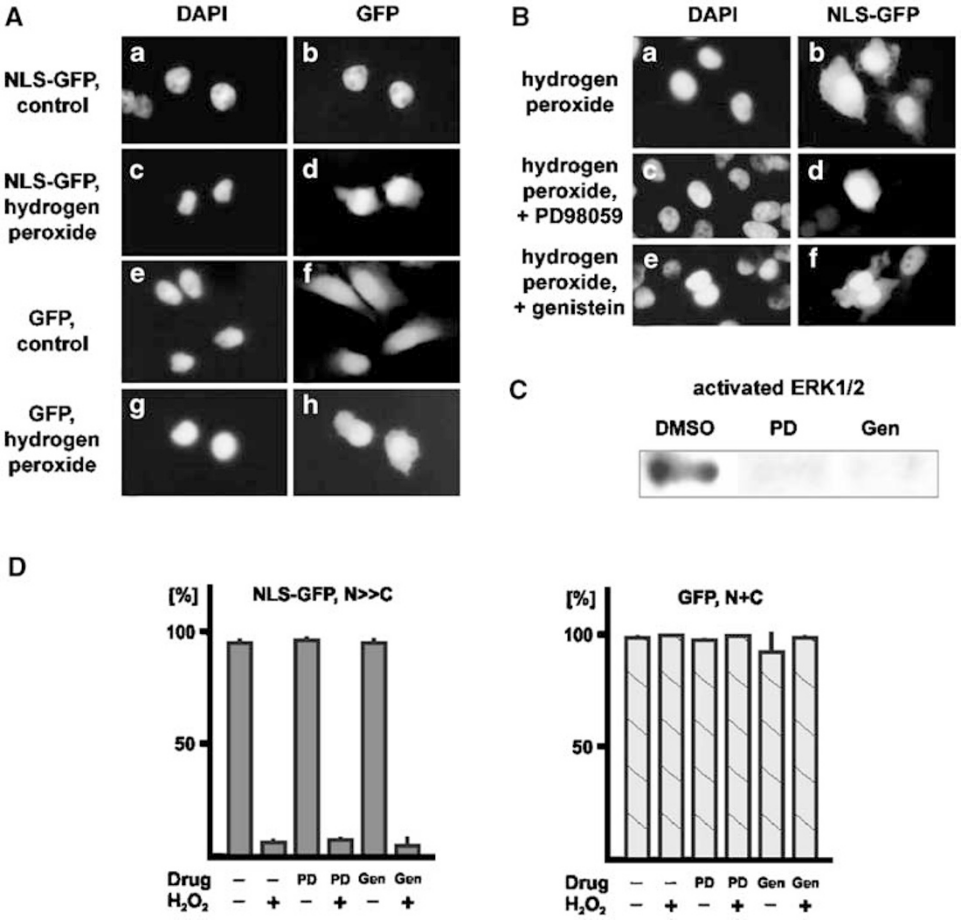

Figure 1 Oxidative stress inhibits classical nuclear import in growing HeLa cells; MAPK inhibitors do not abolish this import inhibition. Treatment with 10 mM hydrogen peroxide was carried out in growth medium for 1 h at 37°C. Control samples were incubated under identical conditions with the omission of oxidant. (**A**) Transiently transfected HeLa cells synthesizing NLS-GFP (a–d) or GFP (e–h) were incubated under nonstress conditions (control) or exposed to hydrogen peroxide. Cells were fixed and reporter proteins were localized by fluorescence microscopy. Nuclei were visualized with DAPI. (**B**) HeLa cells synthesizing NLS-GFP were incubated with hydrogen peroxide in the absence (a, b) or presence of PD98059 (c, d) or genistein (e, f). NLS-GFP and nuclei were detected as in part (**A**). (**C**) HeLa cells were exposed to hydrogen peroxide after preincubation with the solvent DMSO, PD98059 or genistein as indicated. DMSO or inhibitors were also present during exposure to oxidative stress. Equal amounts of protein were separated by SDS-PAGE, and ERK1/2 activation was detected by Western blotting with antibodies specific for dually phosphorylated ERK1/2. (**D**) Quantitation of the effect of hydrogen peroxide on NLS-GFP and GFP localization. Transiently transfected HeLa cells were monitored for the distribution of reporter proteins in nuclei ($N>>C$) or nuclei and cytoplasm ($N+C$). The inhibitors PD98059 (PD) or genistein (Gen) were present as indicated. Each bar represents the mean of results for three independent experiments and the standard deviation. At least 200 transfected cells were evaluated for each experiment

these results, we have chosen 10 mM hydrogen peroxide for our further experiments, as this treatment resulted only in partial mislocalization of the reporter protein to the cytoplasm. HeLa cells exposed to 10 mM hydrogen peroxide frequently changed their shape. This is caused by a reorganization of the F-actin cytoskeleton, which became obvious upon staining with FITC-phalloidin (data not shown). Ultimately, the treatment with hydrogen peroxide is toxic, as many of the cells detached from the surface. Furthermore, when stressed cells were incubated in fresh medium for 24 h, more than 70% of the cells exposed to hydrogen peroxide underwent apoptosis, as evident by TUNEL staining. By contrast, unstressed cells did not score positive in the TUNEL assay (data not shown).

As described above, upon hydrogen peroxide exposure NLS-GFP was detected in the cytoplasm (Figure 1A, d; 1B, b), whereas it was restricted to nuclei in unstressed cells (Figure 1A, b). To quantify the effect of hydrogen peroxide on the distribution of reporter proteins, their localization was assigned to the nuclei (Figure 1D, $N>>C$) or the nuclei and cytoplasm ($N+C$). Under the conditions used in our studies, NLS-GFP was restricted to the nuclei in more than 90% of the control cells. However, after treatment with hydrogen peroxide, in most of the cells NLS-GFP was also detected in the cytoplasm (Figure 1D).

It should be noted, however, that stressed cells did not necessarily equilibrate the reporter protein. Fluorescent signals for nuclei were brighter than for the cytoplasm, demonstrating that the transport substrate relocated only partially (Figure 1A, d; 1B, b, d, f).

Taken together, the data in Figure 1 show that transport of the classical nuclear import substrate NLS-GFP in growing HeLa cells is rapidly inhibited by severe oxidative stress induced by hydrogen peroxide.

Nuclear import inhibition by hydrogen peroxide cannot be abolished by MAPK inhibitors

Hydrogen peroxide has been reported to activate the MAPK ERK2, and changes in ERK2 activity can alter *in vitro* nuclear import in semi-permeabilized smooth muscle cells.[9] We therefore tested whether PD98059, a compound that abolishes the activation of ERK1/2 by inhibiting the upstream kinase MEK, and genistein, a more general inhibitor of MAPKs, interfere with classical import inhibition. To this end, HeLa cells were pretreated with either drug under conditions known to block the activation of ERK1/2.[10] As shown in Figure 1C and published previously,[10] the inhibitor concentrations used in our experiments prevented stress-induced phosphorylation and thereby activation of ERK1/2 in HeLa cells. However, neither PD98059 nor genistein were able to abolish the inhibitory effect of hydrogen peroxide on classical nuclear protein import (Figure 1B, d, f). Moreover, quantitative analysis of the NLS-GFP distribution in stressed cells showed that the effect of hydrogen peroxide was not changed by the treatment with MAPK inhibitors (Figure 1D). Likewise, PD98059 or genistein did not alter classical nuclear protein import in unstressed cells or the distribution of GFP under control and stress conditions (Figure 1D).

Oxidants redistribute nuclear transport factors

The soluble factors Ran and importin-β as well as the nucleoporin Nup153 are essential components of the classical nuclear import apparatus (reviewed in Stochaj and Rother,[11] Görlich and Kutay[12] and Künzler and Hurt[15]). Importantly, hydrogen peroxide treatment of HeLa cells interferes with the formation of the Ran concentration gradient (Figure 2A, d; 2B). This collapse of the Ran concentration gradient was not prevented by PD98059 or genistein (Figures 2B, 4). In unstressed cells importin-β is associated with nuclei, where it accumulates at the nuclear envelope (NE) (Figure 2A, f). In addition, a portion of importin-β can also be detected in the cytoplasm of control cells. However, after exposure to hydrogen peroxide, importin-β becomes confined to the nucleus and no longer accumulates at the nuclear periphery (Figure 2A, h). As observed for Ran, incubation of stressed cells with PD98059 or genistein does not prevent the redistribution of importin-β (Figure 2B). Like Ran and importin-β, Nup153 relocates within cells treated with hydrogen peroxide. Concentrated at the nuclear periphery in unstressed cells, Nup153 redistributes throughout the nucleus after exposure to hydrogen peroxide (Figure 2A, j, l). Since the antibody used for the detection of Nup153 also recognizes several proteolytic products of the nucleoporin, it is possible that intact Nup153, its degradation products or both were redistributed upon oxidant treatment. Relocation of proteins in hydrogen peroxide stressed cells is not a general effect; for instance, lamin B or hsc70 localization was not altered after incubation with this oxidant (Figure 3C and data not shown).

The relocation of nuclear transport factors cannot be ascribed to a simple permeabilization of the NE. We have tested the intactness of the NE by two independent assays. First, cells were transiently transfected with DNA encoding the cytoplasmic reporter protein GFP-β-galactosidase. When cells were treated with oxidant, GFP-β-galactosidase did not enter the nucleus at concentrations of 10 mM or lower (Figure 3A, b, d), demonstrating that the NE is still a barrier for macromolecules. Second, hydrogen peroxide-treated cells were semi-permeabilized with digitonin and incubated with antibodies against lamin B. If the NE is not intact, antibodies will have access to the nuclear lamina. However, this was not observed (Figure 3C, b, f). By contrast, permeabilization with the detergent Triton X-100 allowed antibodies to bind to lamin B (Figure 3C, d, h). Taken together, these results show that the redistribution of nuclear transport factors takes place when the NE and the NPCs do not permit free diffusion of macromolecules.

Hydrogen peroxide could inhibit classical protein import for several reasons. For instance, RanGTP is required in the nucleus to support classical and several nonclassical nuclear trafficking pathways, and dissipation of the Ran concentration gradient may affect these transport routes. In addition, oxidants could change the ratio of RanGTP/RanGDP, thereby interfering with nuclear transport. Like Ran, importin-β, a subunit of the classical NLS-receptor, and Nup153, the termination site for classical nuclear import, redistribute in cells treated with hydrogen peroxide. As such, the carrier importin-β was confined to the nucleus and its NE

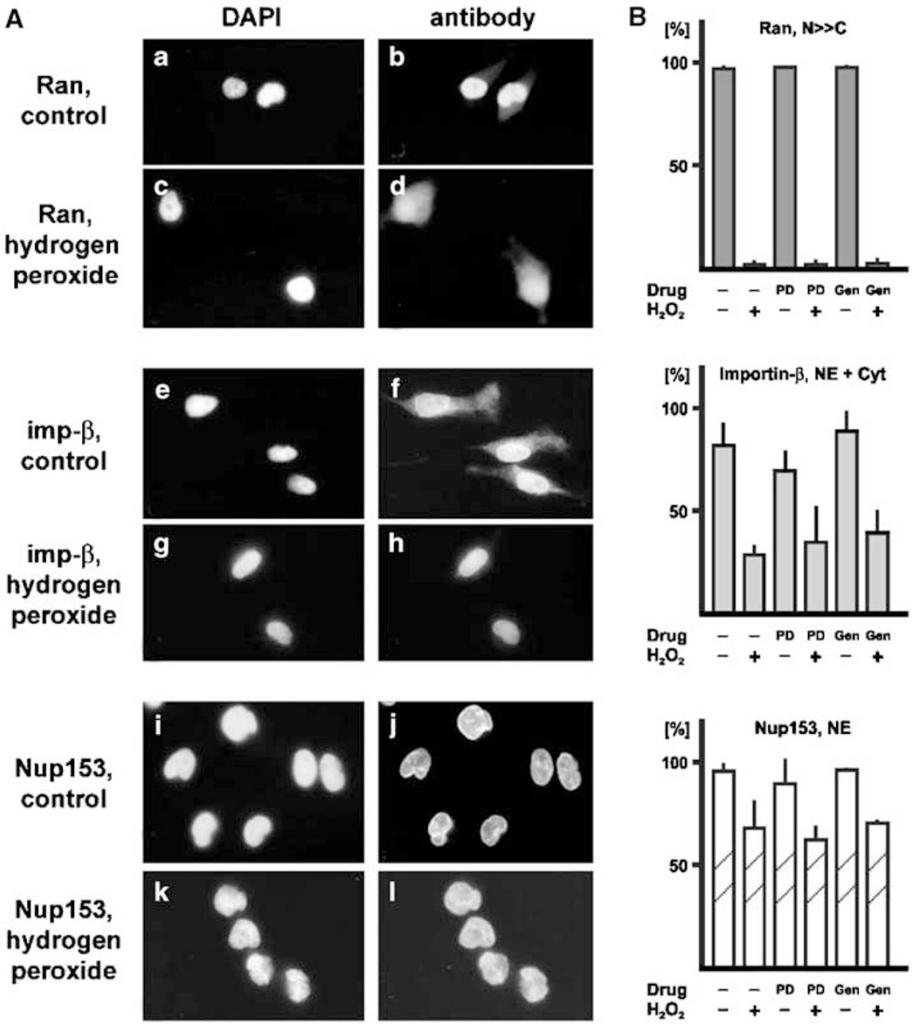

Figure 2 Effect of oxidative stress on the localization of Ran, importin-β, and Nup153. (**A**) Control HeLa cells or cells stressed with hydrogen peroxide as in Figure 1 were used to locate Ran (a–d), importin-β (e–h) and Nup153 (i–l) by immunofluorescent staining. Nuclei were stained with DAPI. (**B**) The distribution of Ran, importin-β and Nup153 was quantified for three independent experiments. For each result, the localization of transport factors was determined in at least 200 cells. The mean values and standard deviations are shown for each group. Changes in Ran nuclear accumulation ($N>>C$), importin-β location at the nuclear envelope and in the cytoplasm ($NE+Cyt$) and the continuous staining of the NE with anti-Nup153 antibodies were monitored

accumulation was reduced. Importin-β has been shown to remain close to the NPC upon completion of nuclear import.[16] It is believed that the carrier will be exported subsequently to the cytoplasm to participate in a new cycle of nuclear import. Hydrogen peroxide treatment could abolish or reduce importin-β exit from the nucleus for different reasons. First, as the Ran concentration gradient collapses, GTPase levels in the nucleus are decreased and may be insufficient to support importin-β export. Second, the association of importin-β with the NE is diminished, presumably because its association with NPCs has been altered. It is clear from our experiments that the NPC organization is modified in cells treated with hydrogen peroxide. In particular, Nup153, located at the nuclear basket in unstressed cells, redistributes in part to the nuclear interior. Furthermore, Nup153 is degraded in stressed cells (see below). Both the relocation and the proteolysis of Nup153 can be expected to affect the functional organization of the nuclear basket and thereby nuclear import and export reactions.

The localization of Ran is more sensitive to oxidative stress than classical nuclear import

Classical nuclear protein import was not efficiently inhibited by hydrogen peroxide concentrations lower than 10 mM (Figures 2, 4). Moreover, NLS-GFP only partially redistributed to the cytoplasm (see above), whereas the Ran concentration gradient was completely abolished under these conditions (Figures 2, 4). This suggests that the Ran concentration gradient is particularly sensitive to oxidative stress. When growing cells were exposed to lower levels of hydrogen peroxide, we detected elevated amounts of Ran in the cytoplasm at concentrations as low as 0.5 mM (Figure 4a).

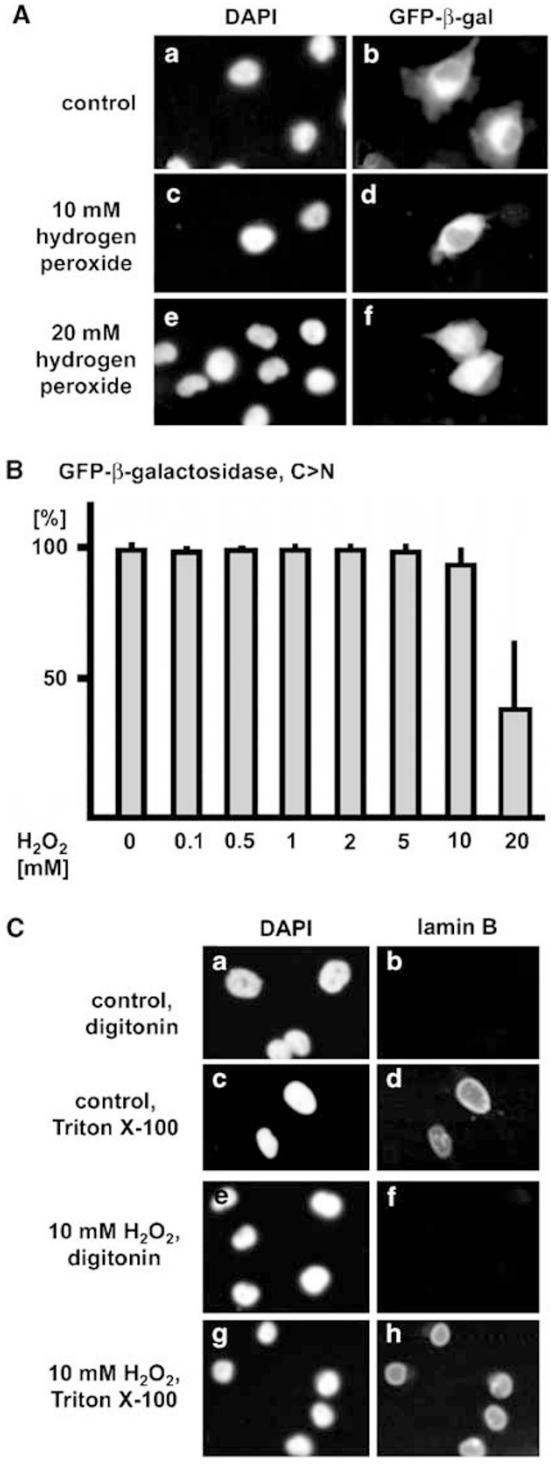

Figure 3 The NE remains intact in HeLa cells treated with hydrogen peroxide. (**A**) HeLa cells transiently synthesizing GFP-β-galactosidase were treated with different concentrations of hydrogen peroxide for 1 h at 37°C. After stress exposure, cells were immediately fixed, stained with DAPI and inspected by fluorescence microscopy. (**B**) At least 100 transfected cells were monitored for each concentration of the oxidant. The figure shows the results (means and S.D.) of three independent experiments. (**C**) Non-transfected HeLa cells were treated with hydrogen peroxide, fixed and permeabilized with digitonin or Triton X-100 as indicated. Cells were incubated with antibodies against lamin B, and primary antibodies were visualized with FITC-conjugated secondary antibodies (Materials and Methods)

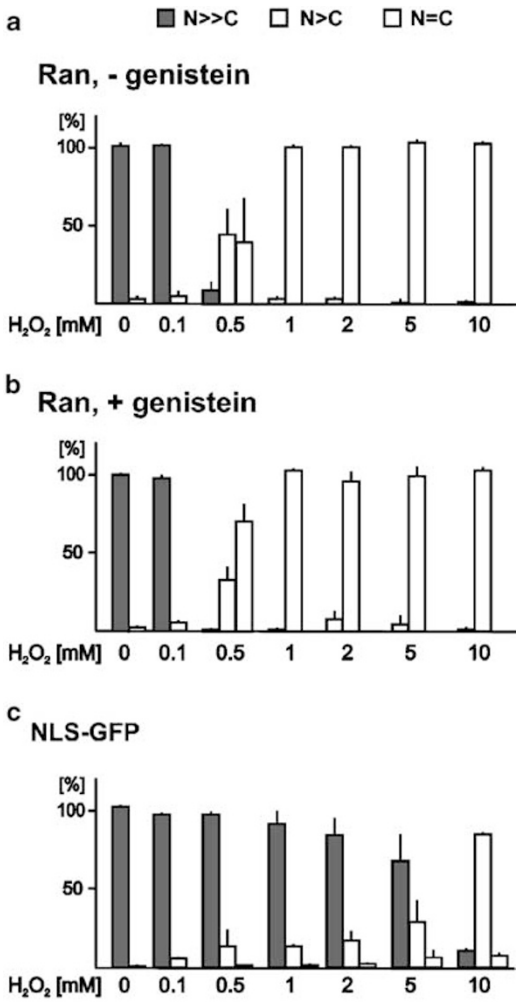

Figure 4 Sensitivity of the Ran concentration gradient and classical nuclear import to hydrogen peroxide. (**a**) Nontransfected HeLa cells were incubated for 1 h at 37°C with different concentrations of hydrogen peroxide. Cells were fixed and Ran was localized by indirect immunofluorescence. (**b**) The same experiment as in part (**a**) was carried out in the presence of genistein. (**c**) HeLa cells synthesizing NLS-GFP were treated with hydrogen peroxide and the reporter protein was located by fluorescence microscopy. The experiments were carried out three times; the distribution of Ran or NLS-GFP was determined for at least 100 cells in each experiment. Parts (**a**)–(**c**) of the figure show the means and the standard deviations for different concentrations of hydrogen peroxide

This redistribution could not be prevented by genistein, and the differences between controls and genistein-treated cells were not statistically significant. Surprisingly, elevated cytoplasmic levels of NLS-GFP were only detected for few cells at 0.5 mM hydrogen peroxide (Figure 4c). This suggests that even though the Ran concentration gradient had collapsed, classical nuclear transport was not drastically affected.

Hydrogen peroxide depletes cellular RanGTP pools

Like other stresses, hydrogen peroxide treatment may reduce the intracellular concentration of ATP, which could subse-

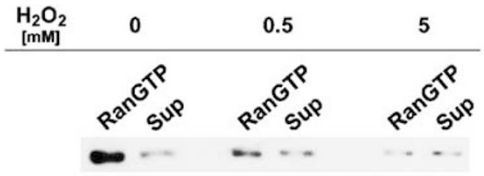

Figure 5 Hydrogen peroxide treatment reduces RanGTP levels. RanGTP was isolated with GST-RBD from controls and cells treated for 1 h at 37°C with different concentrations of hydrogen peroxide. Equal amounts of protein were used as starting material for control and stressed cells. Protein affinity purified with GST-RBD/glutathione-sepharose (RanGTP) and unbound material (Sup) were separated side-by-side, followed by Western blotting with Ran-specific antibodies. For control and stress conditions, the unbound material represents 3% of the affinity-purified protein

quently alter the GTP/GDP ratio. Changes in GTP/GDP may then decrease the levels of RanGTP and favor the formation of RanGDP, a process that will interfere with Ran-dependent trafficking across the NPC. To test this hypothesis, we isolated RanGTP from control and stressed cells with a GST fusion protein that contains the Ran-binding domain (RBD) of RanBP1, referred to as GST-RBD.[17] Crude cell extracts containing the same amount of protein were used as starting material for the purification of RanGTP as recently described,[18] and the isolation of RanGTP was monitored by Western blotting with Ran-specific antibodies. Since hydrogen peroxide treatment induces the degradation of Ran (Figures 7–9 below), we compared the amount of Ran that could be affinity-purified with GST-RBD (Figure 5, RanGTP) to the GTPase that failed to associate with GST-RBD (Figure 5, Sup). As shown in Figure 5, upon exposure to hydrogen peroxide, the relative levels of RanGTP that could be isolated from stressed cells were drastically reduced. A decrease of the ratio RanGTP/RanGDP was already obvious after 1 h exposure to 0.5 mM hydrogen peroxide and became even more pronounced at higher concentrations of the oxidant (Figure 5 and data not shown).

As a consequence of RanGTP depletion induced by oxidative stress, a variety of cellular processes that depend on RanGTP, such as nuclear import and export, will be affected. Furthermore, since RanGDP is unlikely to be retained in the nucleus, changes in the nucleocytoplasmic Ran concentration gradient can also be expected. Ultimately, this will increase cytoplasmic concentrations of the GTPase. Surprisingly, although RanGTP levels were reduced by hydrogen peroxide treatment, classical nuclear transport was not completely abolished under these conditions. One possible explanation for this result could be that even under stress conditions sufficient RanGTP persists in the nucleus to promote NLS-GFP import.

Oxidative stress reduces the import of Ran into the nuclei

In addition to changes in the RanGTP availability, failure to import Ran into the nuclei could also contribute to the relocation of the GTPase to the cytoplasm of hydrogen peroxide-treated cells. NTF2 is the carrier that translocates RanGDP across the NE, and *in vitro* systems have been developed to study this process.[19,20] To address the effect of oxidative stress on Ran nuclear import, we have tested the accumulation of tetramethylrhodamine-labeled RanGDP (TMR-Ran) in semi-intact cells. Under non-stress conditions, TMR-Ran accumulated rapidly in the nucleus and nucleolus if cells were provided with reticulocyte lysate, but treatment with oxidant reduced the capacity of semi-permeabilized cells to concentrate TMR-Ran in the nuclei (data not shown). These results suggested that components of semi-intact cells are sensitive to stress, and we further tested whether hydrogen peroxide also affects cytosolic factors that are required for Ran nuclear import. To this end, we prepared cytosol from control and oxidant-treated HeLa cells, which was combined with untreated or stressed semi-intact cells (Figure 6A). Control cells efficiently imported TMR-Ran when supplied with unstressed cytosol, whereas stressed semi-intact cells displayed increased levels of TMR-Ran in the cytosol. Furthermore, treatment of semi-intact cells with hydrogen peroxide abolished the concentration of TMR-Ran in nucleoli (Figure 6A, d). By contrast, addition of stressed cytosol to untreated semi-intact cells had no drastic effect on the distribution of Ran (Figure 6A, f). If stressed cytosol was combined with stressed semi-intact cells, results were similar to what we observed for the combination of stressed semi-intact cells and untreated cytosol (Figure 6A, compare d and h).

In summary, *in vitro* experiments indicate that Ran nuclear accumulation is reduced, but not abolished, in HeLa cells that have been exposed to severe oxidative stress. This deficiency can be attributed to stress-induced changes of semi-intact cells. As Ran import into nuclei was not prevented by oxidant treatment of cytosol, the source of NTF2 *in vitro*,[19] it is unlikely that the function of NTF2 as a nuclear carrier of the GTPase is abolished by hydrogen peroxide.

NTF2 associates with NEs in hydrogen peroxide-treated cells

Since Ran import was less efficient upon exposure to hydrogen peroxide and NTF2 is the nuclear transporter of Ran, it was important to determine whether NTF2 association with the NE is sensitive to oxidants. Although hydrogen peroxide treatment of cytosol did not prevent Ran nuclear import (Figure 6A), it was possible that stress-induced changes at the NPC prevent the carrier from binding to the NE. Therefore, the association of TMR-labeled GST-NTF2 with nuclei was measured under control and stress conditions using established procedures.[21] After treatment with 0.5, 5 or 10 mM hydrogen peroxide for 1 h, cells were semi-permeabilized and binding of TMR-GST-NTF2 to NEs was tested (Figure 6B and data not shown). TMR-GST-NTF2 accumulation at the nuclear periphery was detected both in the presence and absence of purified wild-type Ran as previously reported.[21] In control experiments, TMR-GST did not associate with semi-intact cells under any of the conditions tested (Figure 6B, j, l and data not shown). These experiments revealed that NTF2 associates with the nuclear membrane in control and stressed cells, suggesting that oxidants did not prevent the interaction between NTF2 and the NPC.

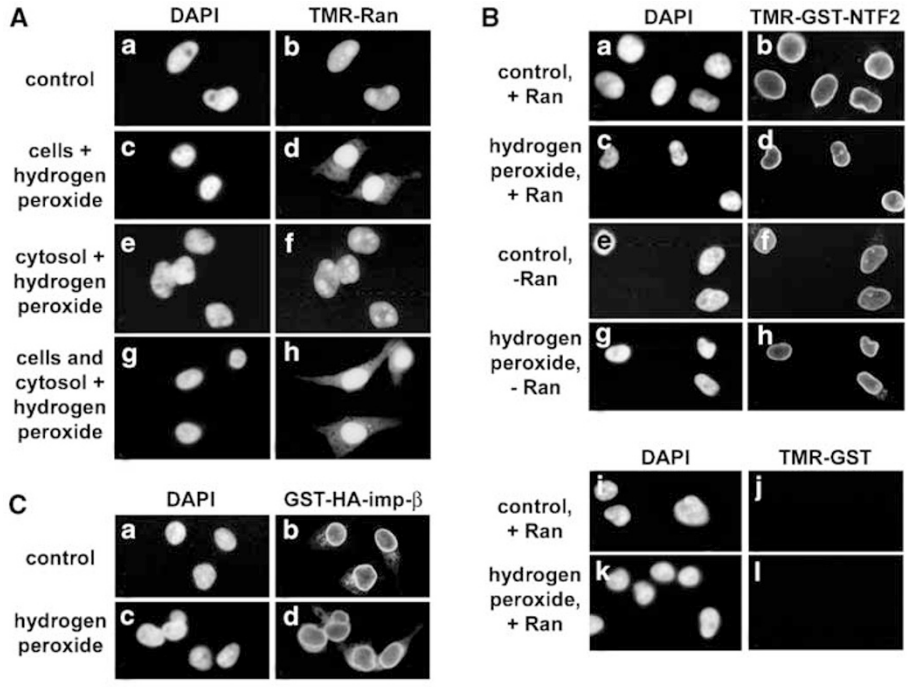

Figure 6 Effect of severe oxidative stress on Ran nuclear import, NTF2 binding to NEs and importin-β nuclear trafficking. For oxidant exposure, cells were incubated with 10 mM hydrogen peroxide for 1 h at 37°C. (**A**) Nuclear import of TMR-RanGDP was analyzed *in vitro* with HeLa cytosol and semi-intact cells. Cytosol and semi-permeabilized cells were prepared from controls or oxidant treated samples as indicated in the figure. (**B**) Binding of TMR-GST-NTF2 (a–h) or TMR-GST (i–l) was tested with semi-intact cells that were unstressed or pretreated with hydrogen peroxide. Unlabeled wild-type Ran was present for the experiments shown in panels (a–d) and (i–l). (**C**) Binding of the classical nuclear import complex importin-α/β/cargo was monitored in unstressed and oxidant treated semi-intact cells. GST-HA-importin-β was localized with antibodies against the HA tag

Nevertheless, Ran is at least in part mislocalized to the cytoplasm under these stress conditions and nuclear import of the GTPase is less efficient when compared to unstressed cells (Figures 2, 6A). There are several possible interpretations of these data: (a) the interaction between Ran and NTF2 is reduced in stressed cells. (b) Oxidants may chemically modify the complex RanGDP/NTF2, preventing it from docking at the NPC. (c) The translocation of RanGDP/NTF2 across the NPC is altered by oxidants. At present, we cannot rule out that hydrogen peroxide interferes with the RanGDP/NTF2 association or docking of the *stressed* RanGDP/NTF2 complex at the nuclear periphery. However, the observation that purified wild-type Ran combined with unstressed cytosol fails to be efficiently imported into the nuclei of stressed semi-intact cells (Figure 6A) indicates that translocation of the GTPase across the NPC is impeded by hydrogen peroxide.

Oxidative stress reduces docking of importin-β at the NE

We further tested whether binding of the import complex importin-α/β/cargo to the nuclear periphery was altered by hydrogen peroxide. To this end, semi-intact cells were incubated with GST-HA-tagged importin-β and the carrier was located with antibodies against the HA tag (Figure 6C). As expected, in semi-intact unstressed cells, importin-β accu-

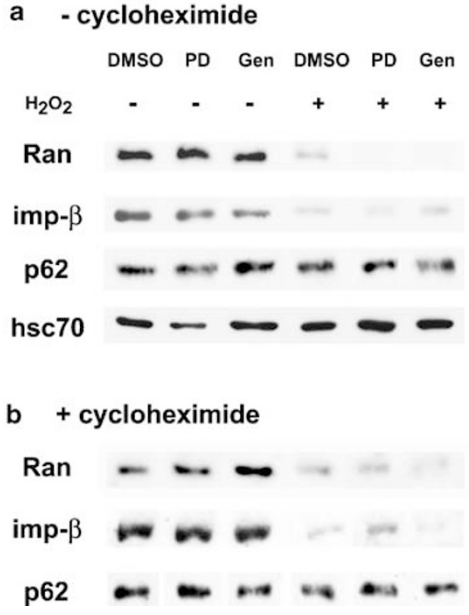

Figure 7 Degradation of Ran, importin-β, p62 or hsc70s in response to hydrogen peroxide treatment. (**a**) Equal amounts of protein from unstressed or stressed cells (1 h, 10 mM hydrogen peroxide, 37°C) were probed by Western blotting with antibodies against Ran, importin-β, p62 and hsc70s. Cells were incubated with the solvent DMSO, PD98059 (PD) or genistein (Gen) as indicated. (**b**) The same experiment was carried out with 100 μg/ml cycloheximide, which was present during stress exposure

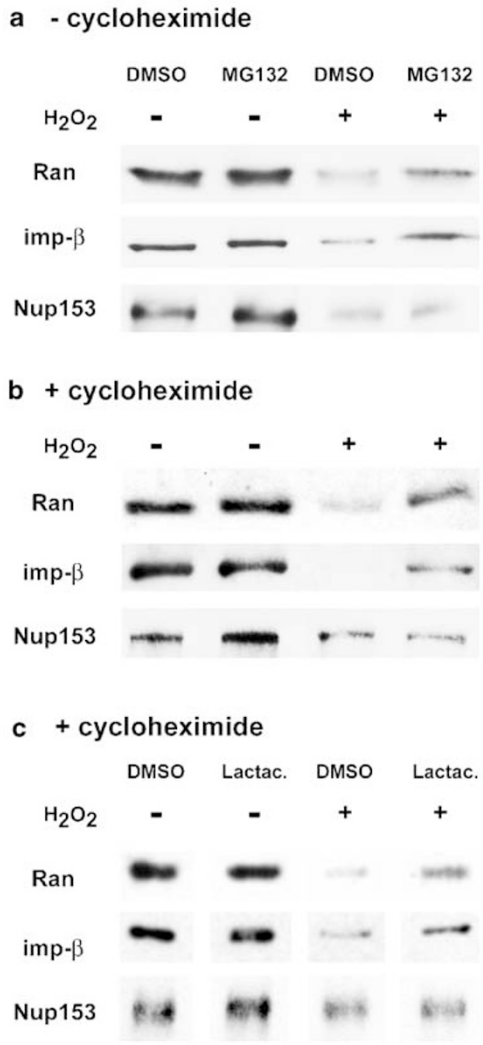

Figure 8 Role of the proteasome in stress-mediated degradation of Ran, importin-β and Nup153. For stress exposure, cells were treated with 10 mM hydrogen peroxide for 1 h at 37°C. Equal amounts of protein were separated side-by-side for each sample and analyzed by Western blotting with antibodies against Ran, importin-β and Nup153. (**a**) Cells were incubated with the solvent DMSO or the inhibitor MG132 as shown in the figure. (**b**) HeLa cells were stressed with hydrogen peroxide in the presence of cycloheximide and MG132. (**c**) HeLa cells preincubated with DMSO or lactacystin were exposed to hydrogen peroxide in the presence of cycloheximide (100 μg/ml)

mulated at the NE.[22] This docking reaction was diminished by oxidative stress, and elevated levels of importin-β remained in the cytoplasm (Figure 6C, d). Nevertheless, even upon hydrogen peroxide exposure, importin-β was able to concentrate at the nuclear periphery, albeit with reduced efficiency.

In summary, severe oxidative stress decreased, but did not prevent, docking of the complex importin-α/β/cargo at the NE. Since importin-β, importin-α or the cargo used in these experiments had not been exposed to stress, we conclude that components of the semi-intact cells are sensitive to hydrogen peroxide, and nucleoporins are possible candidates to be affected by stress.

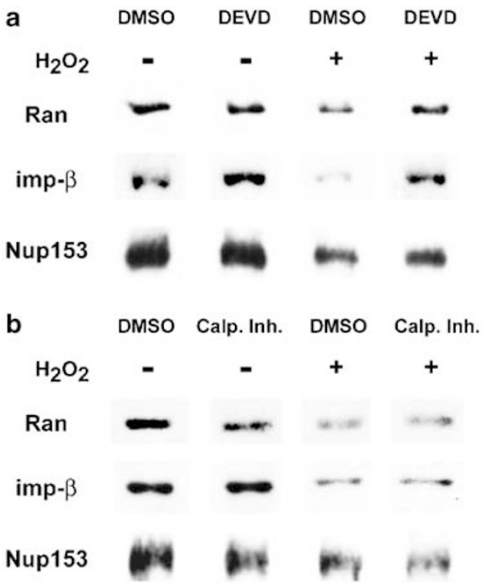

Figure 9 Caspase but not calpain inhibition reduces the degradation of Ran and importin-β upon hydrogen peroxide stress. (**a**) HeLa cells were pretreated with Ac-DEVD-CHO (DEVD) or (**b**) calpain inhibitor I (Calp. Inh.) for 2 h prior to the addition of 10 mM hydrogen peroxide and cycloheximide. Cells were exposed to stress for 1 h at 37°C and cell extracts were subsequently analyzed by Western blotting for the presence of nuclear transport factors, as described for Figure 8**c**

Hydrogen peroxide treatment induces the rapid degradation of Ran, importin-β and Nup153, but not of the nucleoporin p62

Exposure to stress may change the turnover of proteins, and we have analyzed the levels of Ran, importin-β and Nup153 in cells exposed to oxidant. Treatment with hydrogen peroxide reduced the concentration of Ran, importin-β and Nup153 (Figures 7–9). With the antibodies used in our experiments, we did not detect proteolytic products for Ran or importin-β by Western blotting, whereas monoclonal antibody SA1 recognized several degradation products. However, none of these Nup153-derived fragments accumulated upon severe oxidative stress (not shown).

The decrease in Ran, importin-β and Nup153 concentration was not abolished by PD98059 or genistein, and was consistently observed in the presence of MAPK inhibitors (Figure 7). In contrast, levels of hsc70, another soluble factor required for classical nuclear import, the nucleoporin p62 and lamin B were not altered drastically after exposure to hydrogen peroxide under identical conditions (Figure 7 and data not shown). To determine whether hydrogen peroxide diminished the concentration of transport factors by inhibition of their *de novo* synthesis, HeLa cells were incubated with cycloheximide during stress exposure. However, addition of cycloheximide gave similar results, indicating that hydrogen peroxide triggered the degradation of nuclear transport factors (Figure 7b).

Taken together, our data suggest that several nuclear transport factors are particularly prone to degradation when cells are exposed to hydrogen peroxide. Moreover, inhibition of ERK1/2 activation did not abolish their degradation.

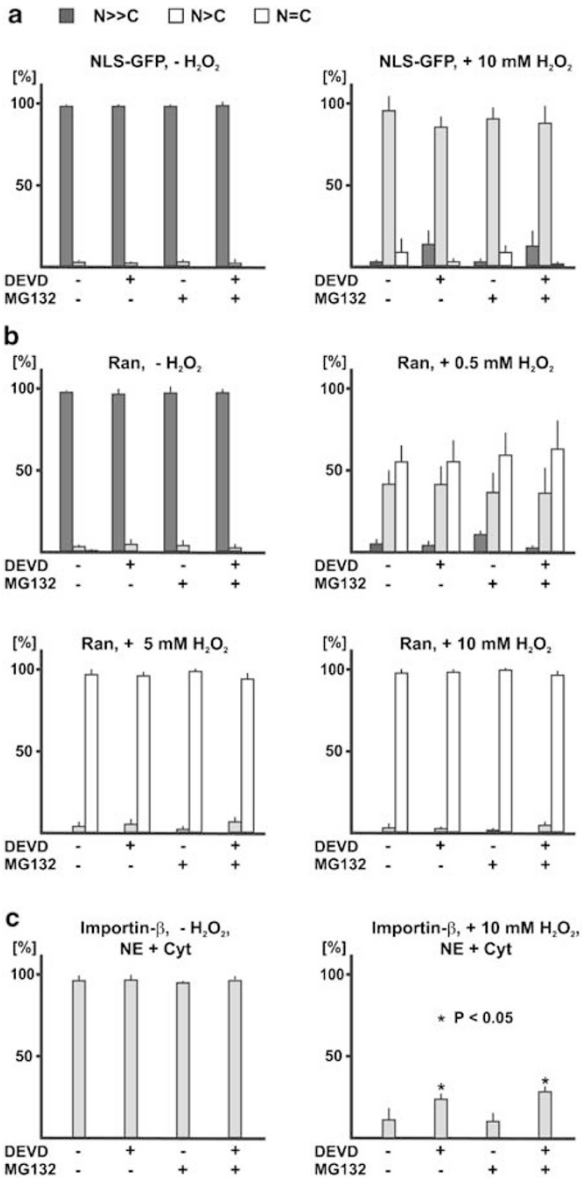

Figure 10 Caspase inhibitors do not abolish the inhibition of classical nuclear import or Ran relocalization, but partially restore the distribution of importin-β. HeLa cells were treated for 1 h at 37°C with different concentrations of hydrogen peroxide. DEVD or MG132 were present for 1 h prior to stress exposure and during the entire stress period. Control cells were incubated with the solvent DMSO only; all of the samples contained the same amount of solvent. Cells were fixed immediately upon oxidant treatment. At least 100 cells were monitored in each of three independent experiments for all of the different experimental conditions shown in the figure. (**a**) Classical nuclear import was monitored in transiently transfected cells synthesizing NLS-GFP, as described for Figure 1. (**b**) Ran was localized by indirect immunofluorescence as in Figure 4. (**c**) Importin-β was located in controls and cells exposed to 10 mM hydrogen peroxide. Incubation with DEVD or DEVD/MG132 significantly increased the number of cells that accumulated importin-β at the NE and also showed cytoplasmic localization of the carrier ($P<0.05$)

In cells treated with hydrogen peroxide, Ran and importin-β degradation is reduced by proteasome inhibitors

Both the 26S and 20S proteasome complexes contribute to the degradation of proteins. In particular, the nuclear 20S proteasome complex may play a role in the proteolysis of nuclear transport factors in stressed cells, as nuclear proteasome activity can be increased by oxidants.[23] Consistent with this hypothesis, MG132, a potent inhibitor of the mammalian proteasome, reduced Ran and importin-β proteolysis in stressed cells, but did not alter Ran or importin-β levels in control cells (Figure 8a, b). By contrast, under the same conditions, MG132 did not protect Nup153 against degradation, suggesting a mechanism independent of the 20S proteasome.

As MG132 may affect proteases other than the proteasome, we have further tested the effect of lactacystin, a distinct proteasome inhibitor, for which we obtained results similar to those described for MG132 (Figure 8c). Degradation of proteins via the proteasome may occur in an ATP/ubiquitin-dependent or -independent fashion. In general, ubiquitination seems to be repressed in cells stressed with oxidants, making ATP/ubiquitin-dependent protein degradation via the 26S proteasome unlikely.[24] Indeed, our data demonstrate that Ran or importin-β ubiquitination is not increased in oxidant-treated cells (see below), in line with ATP/ubiquitin-independent proteolysis. This is consistent with the observation that the 20S but not the 26S proteasome activity is upregulated in cells treated with hydrogen peroxide.[7] As stress-induced degradation of Ran and importin-β can be decreased by MG132 and lactacystin, we conclude that their proteolysis in cells treated with oxidants is partially mediated by the 20S proteasome complex.

Inhibition of caspases diminishes hydrogen peroxide-induced proteolysis of Ran and importin-β

Since HeLa cells underwent apoptosis 24 h after treatment with hydrogen peroxide, we tested whether caspases contribute to the degradation of nuclear transport factors. Proteins of the NPC and the NE have been shown previously to be targets of caspases in apoptotic cells.[25–28] The membrane-permeable compound Ac-DEVD-CHO efficiently inhibits caspase 3. In addition, caspases 6, 7, 8 and 10 can also be affected. The inhibitor reduced the degradation of Ran and importin-β, and the protection against proteolysis was more pronounced than for lactacystin (Figure 9a). For Nup153, the effect of Ac-DEVD-CHO was variable, but always less apparent than for Ran or importin-β.

In addition to the proteasome and caspases, proteases of the calpain family could be involved in the degradation of the transport factors analyzed by us. However, calpain inhibitor I did not alter the proteolysis of Ran, importin-β or Nup153 (Figure 9b). Thus, caspases, but not calpain, are important for the stress-induced proteolysis of Ran and importin-β.

Ran, importin-β and Nup153 are not modified by ubiquitin or SUMO-1 in hydrogen peroxide-treated cells

Ran is essential for classical and nonclassical nuclear transport pathways, the organization of mitotic spindles and reformation of the NE at the end of mitosis.[15,29–33] So far, the

Table 1 Effect of severe oxidative stress on classical nuclear import and nuclear transport factors

Factor or reaction analyzed	Unstressed conditions	Severe oxidative stress
Classical nuclear import of NLS-GFP	Nuclear accumulation	Reduced nuclear accumulation, a portion of NLS-GFP appears in the cytoplasm
Ran localization and stability	Concentrated in nuclei	Ran concentration gradient collapses; Ran degraded by caspases and proteasome
RanGTP		RanGTP/RanGDP ratio decreased
Ran transport *in vitro* into nuclei	Ran efficiently imported into nuclei, accumulation in nucleoli	Ran import with reduced efficiency, no accumulation in nucleoli
NTF2 binding to nuclear envelopes *in vitro*	Purified NTF2 accumulates at the nuclear envelope	No drastic changes, similar to unstressed cells
Importin-β localization and stability	NE, cytosol	NE, reduced amounts in cytosol; importin-β degraded by caspases and proteasome
Docking of import complex importin-α/β/cargo *in vitro*	Importin-α/β/cargo concentrates at the nuclear envelope	Importin-α/β/cargo concentrates at the nuclear envelope, but increased levels of importin-β in the cytoplasm
Nup153	Concentrated at the nuclear periphery	Partial relocation to nuclear interior; degradation

Nuclear accumulation of NLS-GFP, as well as the localization, stability and trafficking of classical nuclear transport factors, was monitored in unstressed controls and cells treated with hydrogen peroxide. See text for details; NE, nuclear envelope

degradation of transport factors under different physiological conditions has not been studied. Exposure to different types of stress can lead to covalent modification of proteins with ubiquitin or SUMO.[34] To determine whether Ran, importin-β and Nup153 are conjugated to ubiquitin or SUMO-1 upon exposure to hydrogen peroxide, we have immunoprecipitated equal amounts of protein from unstressed and stressed cells under denaturing conditions. Immunopurified proteins were then analyzed by Western blotting with antibodies recognizing ubiquitin or SUMO-1. Nuclear transport factors were efficiently purified under these conditions; however, we did not detect them to be ubiquitinated when probed with two different antibodies against ubiquitin. Similarly, two distinct antibodies against SUMO-1 did not reveal a sumoylation of the immunoprecipitated proteins (data not shown). Thus, exposure to hydrogen peroxide does not result in polyubiquitination or modification by SUMO-1 of Ran, importin-β or Nup153. These results are consistent with previous publications for Nup153. Although Nup153 has been shown recently to bind SENP2, a protease that cleaves SUMO-1 modifications, sumoylation of this nucleoporin has not been reported.[35,36] Furthermore, the consensus site for SUMO-1 modifications, ψKxE (with ψ = L, I, V, F; Hay[34]) is not present in human Ran, importin-β or Nup153. In conclusion, severe oxidative stress did not trigger a modification of Ran, importin-β or Nup153 by ubiquitin or SUMO-1.

Effect of protease inhibitors on the oxidant-induced inhibition of classical nuclear import and the relocation of Ran and importin-β

As caspase and proteasome inhibitors reduced the degradation of Ran and importin-β in oxidant-treated cells, we tested whether these compounds also affected nuclear import of NLS-GFP or the redistribution of soluble transport factors in stressed cells. To this end, cells were preincubated with the solvent DMSO, the inhibitors DEVD, MG132 or a combination of DEVD and MG132 (Figure 10); these compounds were also present during the exposure to hydrogen peroxide. Treatment with DEVD or the combination DEVD/MG132 slightly increased the number of stressed cells that efficiently accumulated NLS-GFP in nuclei (Figure 10a, N$>>$C), but these changes were not statistically significant.

In addition to classical nuclear import, the distribution of Ran and importin-β was monitored in control and drug-treated cells (Figure 10b, c). Caspase and proteasome inhibitors did not prevent the relocalization of Ran in stressed cells, and similar results were obtained for different concentrations of hydrogen peroxide (Figure 10b). Unlike Ran, the redistribution of importin-β in stressed cells was partially reversed by DEVD. When DEVD or DEVD/MG132 was present, an increased number of cells concentrated importin-β at the NE and the carrier was also detected in the cytoplasm. By contrast, MG132 alone had no effect (Figure 10c). The changes seen for DEVD or DEVD/MG132 were statistically significant ($P<0.05$).

Taken together, these results suggest that inhibiting the degradation of importin-β by caspases allows cells to retain importin-β in the cytosol, whereas no drastic changes could be observed for Ran or classical nuclear import. One possible interpretation of these data could be that upon severe oxidative stress cytoplasmic importin-β becomes rapidly degraded by caspases. With respect to classical import, however, the protection of importin-β against stress-induced proteolysis is not sufficient to restore the nuclear accumulation of NLS-GFP. This is consistent with the idea that importin-β degradation is not the limiting factor required to re-establish classical transport. Other processes, such as the depletion of RanGTP, might prevent the restoration of the Ran concentration gradient and nuclear trafficking.

Multiple mechanisms contribute to the inhibition of classical nuclear import in oxidant-treated cells

Cells have to cope with various types of insults to prevent or repair stress-induced damage. The effect of stress on protein kinase signaling cascades and the activation of gene transcription has been studied in some detail. However, it has yet to be defined how distinct forms of stress modulate other functions of the nucleus and its organization. To begin to

address this question, we have analyzed in growing HeLa cells and *in vitro* how classical nuclear import and several components of the import apparatus are affected by severe oxidative stress. We have now demonstrated that transport factor relocalization and degradation as well as a depletion of RanGTP are consequences of the exposure to hydrogen peroxide (summarized in Table 1). Each of these changes can be expected to contribute to the inhibition of classical nuclear import. For instance, environmental stress is known to deplete intracellular ATP levels (reviewed in Hardie *et al.*[37]), and we have proposed previously that ATP depletion decreases the concentration of RanGTP, thus favoring the formation of RanGDP.[8] This has recently been confirmed in HeLa cells, for which the reduction of ATP resulted in lower levels of RanGTP.[18] The increased consumption of ATP necessary to repair oxidant-induced damage may transiently alter the homeostasis of ATP production and consumption (reviewed in Dzeja and Terzic[38]). Under these conditions, RanGTP levels could become limiting for the support of nuclear trafficking. Furthermore, the lack of nuclear retention of RanGDP will promote a collapse of the nucleocytoplasmic Ran concentration gradient.

A prominent feature of Ran, importin-β and Nup153 is their increased sensitivity to proteolysis in response to severe oxidative stress. Hydrogen peroxide triggers the oxidation of SH groups and other amino-acid side chains (reviewed in Dean *et al.*[39]), which could target proteins for degradation via the proteasome. It was proposed that protein oxidation increases surface hydrophobicity, thereby enabling the 20S proteasome to recognize oxidized proteins even in the absence of ubiquitination,[24] a process that might take place for Ran and importin-β. By contrast, Nup153 degradation was independent of the proteasome, and inhibition of caspases had variable effects. Other studies have shown Nup153 to be a substrate for caspases in apoptotic cells.[25,27] However, Nup153 does not always undergo degradation during apoptosis[26] and differences are likely to exist for various cell types. These differences may also explain why importin-β and Ran are not degraded upon induction of apoptosis with cisplatin.[26]

Our experiments clearly show that ERK1/2 are not the limiting factors, that alter classical nuclear import in response to severe oxidative stress. As oxidants also activate signaling pathways other than the ERK1/2 cascade, classical nuclear transport inhibition is likely to be more complex in growing mammalian cells than previously reported for *in vitro* studies.[9] As well, cell type-specific differences may explain that in semi-permeabilized smooth muscle cells ERK2 is the critical component that controls classical import after exposure to hydrogen peroxide, whereas transport inhibition in HeLa cells involves multiple mechanisms.

In vitro experiments shown in this study suggest that NPCs are likely to be targets for damage induced by hydrogen peroxide. As such, Ran import into nuclei was reduced if semi-intact cells have been stressed. Furthermore, docking of the classical nuclear import complex at the NE was diminished. The simplest explanation of these results is a change in NPC function, and future studies will have to identify which nucleoporins, in addition to Nup153, are affected by oxidants.

Besides NPCs, several soluble factors required for classical nuclear import are shared with nonclassical trafficking pathways. As such, Ran and its interacting components are essential for transport that is mediated by members of the importin-β family. Changes in Ran localization, concentration and the RanGTP/RanGDP ratio after treatment with oxidants will therefore have a more general effect on nucleocytoplasmic transport of macromolecules. Not only classical transport but also nonclassical import as well as export from the nucleus will be affected. Taken together, our results underscore that severe oxidative stress can modulate nuclear functions on several levels, including the inhibition of nuclear trafficking reactions and the functional organization of NPCs.

Materials and Methods

Growth and stress exposure of HeLa cells

HeLa cells were grown in multiwell chambers as described.[40] At approximately 70% confluency, cells were subjected to 10 mM hydrogen peroxide (or the oxidant concentration given in the figure legends) in growth medium and incubated for 1 h at 37°C. Incubation with the kinase inhibitors PD98059 and genistein was carried out as in Chu *et al.*[10] To test the role of the proteasome, cells were preincubated for 30 min with 10 μM MG132 or for 2 h with 50 μM lactacystin (Calbiochem, San Diego, CA, USA) in growth medium. Cells were pretreated with 15 μM caspase inhibitor (Ac-DEVD-CHO, Calbiochem) or 50 μM calpain inhibitor I (MG101, Sigma, Oakville, ON, USA) 2 h before stress exposure. All inhibitors were present throughout the stress treatment.

Transfection of HeLa cells

HeLa cells were transfected with plasmids encoding the nuclear reporter protein NLS-GFP[10] or plasmid pHM830 coding for GFP-β-galactosidase[41] following standard procedures.[10,40]

Generation of GST-NTF2 and synthesis of fusion proteins in *E. coli*

GST-NTF2 was generated by fusing GST in frame to codon 4 of human NTF2. The correctness of the construct was verified by DNA sequencing. A plasmid encoding GST-HA-importin-β[22] was kindly provided by Dr. Y Yoneda, Osaka. Expression of genes encoding GST- or His6-fusion proteins was induced in bacteria with 0.5 mM IPTG for 2.5 h at 37°C.

Fluorescent labeling of His6-Ran and GST-NTF2

His6-tagged wild-type Ran, GST-NTF2 and GST were synthesized in *E. coli* and affinity purified under native conditions following standard procedures. Purified proteins were dialyzed against 50 mM potassium phosphate, pH 7.0, 2 mM magnesium acetate and concentrated with centrifugal filters (Millipore, Bedford, MA, USA). GDP-loaded Ran was labeled with tetramethylrhodamine-maleimide (TMR-maleimide; Molecular Probes, Eugene, OR, USA) for 3 h on ice essentially as described.[19] GST-NTF2 or GST were labeled overnight on ice. Non-incorporated label was removed by gel filtration using Sephadex G25 (Amersham Biosciences, Piscataway, NJ, USA) equilibrated with Ran import buffer (see below), which was supplemented with 200 μM GDP for the purification of labeled Ran.

Nuclear import of Ran

Nuclear import of fluorescent Ran was tested essentially as in Ribbeck *et al.*[19] In brief, HeLa cells were grown on poly-lysine-coated slides to 70% confluency and semi-permeabilized with 40 μg/ml digitonin in Ran import buffer. Cells were washed once in ice-cold import buffer and nuclear accumulation of Ran in import buffer containing 0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 2 mg/ml BSA and 4 mg/ml reticulocyte lysate, or 3 mg/ml HeLa cytosol was allowed for 3 min at room temperature. Cells were fixed immediately with 3.7% formaldehyde for 20 min at room temperature and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

Binding of NTF2 to NEs

The association of fluorescent GST-NTF2 (TMR-GST-NTF2) or GST (TMR-GST) with nuclear membranes was tested essentially as described.[21] Binding of GST-NTF2 to NEs of semi-intact cells was tested with 0.45 μM TMR-GST-NTF2 (dimer) or TMR-GST combined with an energy-regenerating mix.[21] Wild-type RanGDP was present at 1.15 μM as indicated in Figure 6B. Upon incubation for 10 min at room temperature, cells were fixed for 20 min with 3.7% formaldehyde and nuclei were visualized with DAPI.

Docking of the classical nuclear import complex at nuclear membranes

Binding of purified GST-HA-importin-β was tested in semi-intact cells essentially as described.[22] Importin-α, GST-HA-importin-β and 50 μg/ml SV40-HSA were preincubated for 1 h on ice and centrifuged (5 min, microfuge, 13 000 rpm) before addition to semi-intact cells. After incubation for 20 min at room temperature, excess liquid was removed and cells were immediately fixed with 3.7% formaldehyde in PBS (20 min, room temperature). Importin-β was localized by indirect immunofluorescence with antibodies against the HA tag (see below).

Specificity of antibodies used for immunofluorescence and Western blotting

The following primary antibodies were used: mouse mab414 and monoclonal antibodies to the HA tag (BaBCo, Richmond, CA, USA), goat anti-lamin B (sc-6217, Santa Cruz Biotechnology, CA, USA), goat anti-Ran (sc-1155, sc-1156, Santa Cruz Biotechnology), monoclonal antibody 3E9 against importin-β (Affinity Bioreagents, Golden, CO, USA), monoclonal antibody SPA-815 against hsc70 (StressGen, Victoria, BC, Canada), cell culture supernatant SA1 (Bodoor *et al.*;[42] a generous gift of Dr. B Burke), specific for Nup153, and rabbit polyclonal and monoclonal antibodies to ubiquitin (sc-9133 and sc-8017, Santa Cruz Biotechnology), polyclonal and monoclonal antibodies recognizing SUMO-1 (sc-6375, sc-5308, Santa Cruz Biotechnology). Mab414 binds FXF repeat containing nucleoporins, including Nup153 and p62. Antibodies against lamin B, Ran, importin-β and hsc70 recognize a single band of the expected size on Western blots. Monoclonal antibody SA1 binds to the 180 kDa nucleoporin Nup153 and also to some of its degradation products. The different anti-ubiquitin antibodies bind to several bands for crude extracts upon Western blotting. Activated ERK1/2 was detected with monoclonal antibody E10 (Cell Signaling Tech., Beverly, MA, USA), which is specific for phospho-p44/42 MAPK, phosphorylated in positions thr202 and tyr204. Primary antibodies were diluted as suggested by the suppliers and supernatant SA1 was used undiluted.

Immunofluorescence

All steps were carried out at room temperature. Stressed cells and controls were fixed in 3.7% formaldehyde/PBS for 25 min. Cells were permeabilized in 0.1% Triton X-100 (5 min), blocked for 1 h in PBS/2 mg/ml BSA containing 0.05% Tween 20 and incubated overnight with primary antibodies. Bound primary antibodies were detected with FITC-conjugated anti-rabbit or anti-goat antibodies, or Cy3-conjugated antibodies to mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA). Secondary antibodies were diluted between 1 : 200 and 1 : 250. DNA was visualized with DAPI and samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Cells were analyzed with a Nikon Optiphot at $\times$ 400 magnification and photographed with Kodak T-MAX 400 films. The negatives were scanned and processed with Photoshop 5.5.

Digitonin treatment of HeLa cells for immunofluorescence

Control and stressed HeLa cells were fixed and incubated with 40 μg/ml digitonin in PBS for 3 min on ice. In Figure 3C, cells were incubated with 0.1 μg/ml anti-lamin B antibodies overnight at room temperature. After three washes, samples were incubated with FITC-labeled secondary antibodies, washed and treated with DAPI. All incubations were carried out as described for immunofluorescent staining, except for the omission of Tween 20 in all buffers.

Western Blot analysis

HeLa cells were grown on dishes to about 70% confluency. Upon stress exposure, plates were washed with PBS and stored at -70°C until use. Proteins were solubilized in gel sample buffer and treated as described.[43] Equal amounts of protein were separated for controls and different stress conditions in SDS-PA gels and blotted to nitrocellulose filters. Blots were further processed as previously described.[10,43]

Purification of RanGTP

RanGTP was isolated with GST-RBD essentially as in Schwoebel *et al.*[18] Aliquots of affinity-purified RanGTP and material that did not bind to GST-RBD were separated side-by-side on 12% SDS-PA gels and Ran was detected by Western blotting.

Indirect immunoprecipitation

Protein G-sepharose (Amersham Biosciences) was preloaded with Ran-specific antibodies overnight at 4°C in RIPA-buffer (PBS/1% Triton X-100/0.1% SDS/0.5% sodium deoxycholate/1 mM NaN_3, pH 7.4). Control resin was incubated with RIPA buffer only. For immunopurification of denatured proteins, HeLa cell extracts were prepared in PBS containing 1 mM sodium orthovanadate, 1 mM PMSF and a cocktail of protease inhibitors (aprotinin, antipain, chymostatin, leupeptin and pepstatin, each at 0.1 μg/ml). DNA was sheared by vortexing samples in the presence of glass beads. Proteins precipitated with 5% TCA for 5 min on ice were collected by centrifugation. Sediments were resuspended in RIPA buffer containing inhibitors (see above) and preincubated with protein G-sepharose for 30 min at 4°C. Supernatants obtained after 5 min centrifugation (5000 rpm, microfuge, 4°C) were mixed with control resin or resin loaded with antibodies against Ran, importin-β, or Nup153. After overnight incubation at 4°C with gentle agitation, protein G-sepharose was collected

by centrifugation and washed four times in cold PBS/1 mM NaN_3. Proteins bound to the resin were eluted by boiling in gel sample buffer (10 min at 95°C), followed by Western blot analysis with antibodies against ubiquitin or SUMO-1.

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