Hydrogen Peroxide Inhibition of Nuclear Protein Import Is Mediated by the Mitogen-activated Protein Kinase, ERK2

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Abstract. H_2O_2 alters gene expression in many cell types. Alterations in nuclear import of transcription factors or similar key proteins may be responsible for these changes. To investigate this possibility, a cytosolic nuclear import cocktail was treated with varying $[H_2O_2]$ and used in import assays. H_2O_2 caused a dose- and time-dependent inhibition of import at concentrations as low as 100 µM. Catalase reversed this effect. H_2O_2 treatment of permeablized cells did not affect import, suggesting that H_2O_2 was acting on a cytosolic factor. Treatment of import cocktail with two different free radical generating systems had no effect, but treatment of permeablized cells inhibited import, suggesting H_2O_2 works via a distinct process from hydroxyl or superoxide radicals. Pretreatment of import cocktail with genistein reversed the effect of H_2O_2 on import. Western blotting revealed that H_2O_2 activated ERK2. The specific MEK1/2 inhibitor, PD98059, completely blocked the effects of H_2O_2 on import. Activated ERK2 mimicked H_2O_2's effect on import. Immunocytochemistry revealed that H_2O_2 treatment of whole cells increased cytosolic Ran/TC4 levels, an effect reversible by catalase or PD98059. These data demonstrate that H_2O_2 inhibits nuclear protein import and that this effect is mediated by mitogen-activated protein (MAP) kinase activation, possibly by altering Ran/TC4 function.

Key words: phosphorylation • confocal microscopy • permeablized cells • free radicals • signal transduction

Introduction

Nuclear protein import is the specific, energy-dependent process by which key proteins, such as transcription factors, kinases, DNA binding proteins, and polymerases, enter the nucleus in a coordinated fashion from their synthesis site in the cytoplasm (Agutter and Prochnow, 1994; Csermely et al., 1995; Corbett et al., 1996; Gorlich and Mattaj, 1996; Gorlich, 1997). These factors play critical roles in both proliferation and apoptosis. Many of these factors contain nuclear localization sequences (NLSs)[1] and enter the nucleus through an NLS-dependent process (Csermely et al., 1995; Jans and Hübner, 1996). NLSs do not have consensus sequences, but often consist of a stretch of basically charged amino acids (Goldfarb et al., 1986; Gorlich and Mattaj, 1996). Molecules greater in size than ~40 kD will traverse the nuclear pore very slowly or not at all, but addition of an NLS to a normally cytoplasmic protein causes rapid nuclear uptake of the protein.

The first step of NLS-dependent import is binding of the NLS-bearing protein to the import complex, consisting of the NLS receptor importin α and importin β, which is required for docking of the import complex with the nuclear pore complex (Agutter and Prochnow, 1994; Csermely et al., 1995; Corbett et al., 1996; Gorlich and Mattaj, 1996; Gorlich, 1997). NTF2, a protein factor required for import, also binds to the import complex, as well as Ran/TC4, a small ras-related GTPase (Paschal and Gerace, 1995; Avis and Clarke, 1996). The import complex then docks to the nuclear pore complex, possibly by interaction with RanBP2, a protein located on the cytosolic spindles of the pore (Gorlich, 1997). The next stage of import is transit of the import complex through the pore. This step has long been believed to require GTP hydrolysis by Ran/TC4. This hydrolysis is catalyzed by RanGAP1, a Ran GTPase activating protein, and interference with GTP hydrolysis, either by using nonhydrolyzable GTP analogues or by removing RanGAP1, inhibits import (Schlenstedt et al., 1995; Avis and Clarke, 1996). Recently, however, it has been shown that hydrolysis of GTP by Ran is not strictly

required for import to occur (Schwoebel et al., 1998). Rather, Ran appears to be important for proper cycling of import factors between the cytoplasm and nucleus. The cycling of Ran between a GTP- and GDP-bound state, therefore, appears to play a permissive role for import to occur.

Alteration of rates of nuclear import has the potential to affect cell functioning by altering nuclear partitioning of various proteins, such as transcription factors. Recent studies have demonstrated that import rates of individual proteins may be regulated by phosphorylation of key amino acids upstream from the protein's NLS (Rihs and Peters, 1989; Jans et al., 1991; Rihs et al., 1991; Tagawa et al., 1995; Jans and Hübner, 1996; Hübner et al., 1997), but this only affects import of the phosphorylated protein. It is unknown whether the import process in general, independent of modifications of the imported molecule itself, is regulated. The possibility that such regulation exists is suggested by experiments where nuclear import was abolished by the artificial depletion of perinuclear calcium (Greber and Gerace, 1995; Stehno-Bittel et al., 1995). Furthermore, it has been shown that proliferating cells demonstrate greater rates of nuclear import than quiescent cells (Feldherr and Akin, 1991, 1993).

H_2O_2 is generated by cells in both normal and pathological states. It is a by-product of normal cell aerobic metabolism, and is generated by a variety of cells in processes such as atherogenesis and ischemia (Boveris, 1977; Babior, 1978; Burton et al., 1984; Kloner et al., 1989; Bast et al., 1991; Kaul et al., 1993; Vanden Hoek et al., 1997; Sabri et al., 1998). H_2O_2 has been implicated in both proliferation and apoptosis of smooth muscle cells (Abe et al., 1994; Cantoni et al., 1996; Li et al., 1997; Zafari et al., 1998). In both situations, activation of the mitogen-activated protein (MAP) kinase ERK2 and stimulation of DNA synthesis has been reported as a result of H_2O_2 exposure (Fiorani et al., 1995; Cantoni et al., 1996; Li et al., 1997). In light of these paradoxical findings, it is of interest to determine how H_2O_2 may be exerting its effects on cell functioning.

We examined whether H_2O_2 and free radicals had the capacity to influence nuclear protein import and the mechanism of action involved. We report here that H_2O_2 adversely affects the nuclear import process in a dose- and time-dependent manner. This effect is correlated with the activation of the MAP kinase, ERK2. Furthermore, exogenous activated ERK2 is able to inhibit import similar to the effect of H_2O_2. H_2O_2 causes an increase in cytosolic Ran/TC4 levels. These findings suggest that alterations in MAP kinase activation may have important consequences on movement of proteins into the nucleus. These alterations in import may adversely affect gene expression and other nuclear processes, and may explain, in part, the ability of H_2O_2 to cause apoptosis. These data not only demonstrate the first evidence of a generalized control of import, but also have important implications for cell functioning in situations where H_2O_2 is generated.

Materials and Methods

Materials

DME, FBS, and fungizone were purchased from GIBCO BRL. BODIPY FL-conjugated BSA (BODIPY-BSA) and Alexa488-conjugated secondary antibodies were purchased from Molecular Probes Inc. Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate, excellulose columns, and the SuperSignal kit were purchased from Pierce Chemical Co. Tris-(2-carboxyethyl) phosphine HCl, PD98059, protein–A agarose, nonactivated and activated ERK2 were purchased from Calbiochem-Novabiochem Corp. SpectraPor membrane was purchased from Spectrum. Anti-ACTIVE MAP kinase mAbs were purchased from Promega Corp. Anti-Ran/TC4 mAbs were purchased from Transduction Laboratories. HRP-conjugated secondary antibodies were purchased from BioRad Laboratories. Vectashield was purchased from Vector Laboratories, Inc. All other chemicals were purchased from Sigma-Aldrich Canada, Ltd.

Cell Culture

Vascular smooth muscle cells were obtained from aortic explants of New Zealand white rabbits as described (Saward and Zahradka, 1997). Cells were maintained in DME supplemented with 10% FBS plus 1% fungizone until confluent, then passaged onto glass coverslips in fresh medium. Cells were maintained in 10% FBS/DME + 1% fungizone for 2 d, then switched to a medium containing DME plus 5 μg/ml transferrin, 1 nM selenium, 200 μM ascorbate, 10 nM insulin, 2.5 μM pyruvate plus 1% fungizone for ~5 d. Cells were fed with 10% FBS/DME + 1% fungizone 24–48 h before use.

Nuclear Import Assay

Assays were carried out using the procedure of Adam et al. (1991) with minor modifications. The import substrate used was BODIPY-BSA conjugated to the SV40 large T antigen NLS (CGGGPKKKRKVED). Assay results were measured on a BioRad MRC600UV confocal microscope and analyzed using Molecular Dynamics ImageSpace 3.2.1 software. Multiple fields of cells were visualized per experiment and averaged, so that each data point represents results for ~60–180 cells measured in three to four experiments.

Isolation of Rat Liver Cytosol

A male Sprague-Dawley rat (~250 g) was killed by decapitation and the liver quickly removed into ice-cold STM buffer consisting of 250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, and 1 μM leupeptin. The liver was scissor-minced and washed several times with STM buffer, then homogenized. The homogenate was centrifuged in a Beckman JA-20 rotor at 1,400 g for 10 min at 4°C. The supernatant was removed and centrifuged at 3,300 g for 15 min at 4°C. This supernatant was then centrifuged in a Beckman SW28 rotor at 100,000 g for 1 h at 4°C. The final supernatant was removed as "cytosol". Cytosol was dialyzed overnight through Spectrapor 1 dialysis tubing against multiple changes of import buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA) including 1 mM DTT and 1 μg/ml each of leupeptin, pepstatin A, and aprotinin.

Treatment of Import Cocktail or Permeablized Cells

Import cocktail consisted of import buffer plus 50% rat liver cytosol, 1 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase, and 1 μg/ml each of leupeptin, pepstatin A, and aprotinin. For some experiments, import cocktail was treated with 0.1, 0.5, or 1.0 mM H_2O_2 for 30, 60, or 120 min at 37°C, with or without 0.3 mg/ml catalase. Alternately, import cocktail was treated with 2 mM xanthine plus 0.03 U/ml xanthine oxidase, with 0.1 mM H_2O_2 plus 0.1 mM FeSO₄, or with 40 ng/ml activated ERK2. Some aliquots of import cocktail were also pretreated for 45 min before H_2O_2 treatment with 75 μM genistein or daidzein, or 20 μM PD98059 at 37°C. Controls received import buffer alone. The nuclear import assay or Western blotting was then carried out. For treatment of permeablized cells, aortic vascular smooth muscle cells were permeablized with 40 μg/ml digitonin for 5 min, then treated with 1.0 mM H_2O_2 for 30 min, with 2 mM xanthine plus 0.03 U/ml xanthine oxidase for 10 min, or with 0.1 mM H_2O_2 plus 0.1 mM FeSO₄ for 60 min. Cells were then rinsed briefly with import buffer and nuclear import assays carried out.

Western Blotting

Import cocktail was treated as described above, then run on 10% acrylamide SDS-PAGE gels. Gels were transferred onto nitrocellulose, and the

blots probed with anti-ACTIVE MAPK antibodies. Immunoreactive bands were visualized using a SuperSignal Kit and exposed to Kodak X-OMAT film. Blots were scanned on a Umax Powerlook II scanner and intensity measured using Un-Scan-It Gel software, version 5.1.

Immunocytochemistry

Rabbit aortic smooth muscle cells grown on glass coverslips were treated with 1.0 mM H_2O_2 with or without either 0.3 mg/ml catalase or 20 μM PD98059 as described above. Cells were then washed five times with PBS and fixed in 1% paraformaldehyde in PBS for 15 min at 4°C. Cells were washed again, then permeablized in 0.1% Triton X-100 in PBS for 15 min at 4°C. Cells were washed repeatedly in PBS and incubated with anti-Ran/TC4 antibodies (1:250 dilution) in PBS containing 1% BSA and 0.02% sodium azide overnight at 4°C. Cells were washed 12 times with PBS, then incubated with Alexa$_{488}$-conjugated secondary antibodies (1:1,000 dilution) in PBS plus 1% BSA and 0.02% sodium azide for 90 min at 4°C. After washing three times, cells were mounted onto glass slides with Vectashield as mountant and observed by confocal microscopy. Cytoplasmic fluorescence was measured as described for nuclear fluorescence.

Statistical Analysis

Variation between means was determined by two-tailed Student's t test, or by one-way analysis of variance with Student-Neuman-Keuls post-hoc test with $P < 0.05$ considered statistically significant. Nuclear and cytoplasmic fluorescence values are reported as percentage of control.

Results

H_2O_2 Inhibits Nuclear Protein Import

Typical results of the nuclear import assay used in this study are shown in Fig. 1. The fluorescent import substrate (BODIPY-BSA conjugated to an SV40 NLS) is markedly accumulated in the nucleus after import assay in control cells (Fig. 1 A). The appearance of these cells after import assay is similar to that reported by others (Adam et al., 1991; Gorlich and Laskey, 1995; Moore and Blobel, 1995; Sweet and Gerace, 1995). When the nuclear import cocktail (rat liver cytosol plus import buffer, an energy production system and protease inhibitors) is pretreated for 60 min at 37°C with 1 mM H_2O_2, however, this nuclear fluorescence is substantially reduced (Fig. 1 C). This effect could be attenuated by the inclusion of 0.3 mg/ml catalase, an enzyme that scavenges H_2O_2, during the pretreatment of the cells with H_2O_2 (Fig. 1 D). Treatment of import cocktail with catalase alone had no effect on nuclear protein import (Fig. 1, B and E).

The effect of varying concentrations of H_2O_2 on nuclear import was examined. As shown in Fig. 1 E, pretreatment of import cocktail with concentrations of H_2O_2 as low as 100 μM caused a significant inhibition of nuclear protein import ($\sim$90% of control values), and this effect was enhanced as [H_2O_2] was increased. Inclusion of 0.3 mg/ml catalase during pretreatment was able to completely block this effect with 100 and 500 μM H_2O_2. When [H_2O_2] was increased to 1 mM, import was reduced to $\sim$60% of control values. Inclusion of 0.3 mg/ml catalase significantly improved recovery of import, but was unable to completely reverse the effects of the H_2O_2. Increasing the [catalase] to 1.4 mg/ml caused a further significant increase in import recovery, but was still unable to restore import to control values.

Inhibition of import by H_2O_2 also exhibited time dependency, as shown in Fig. 2. Increasing the time of treatment of import cocktail with H_2O_2 resulted in a steady decline in
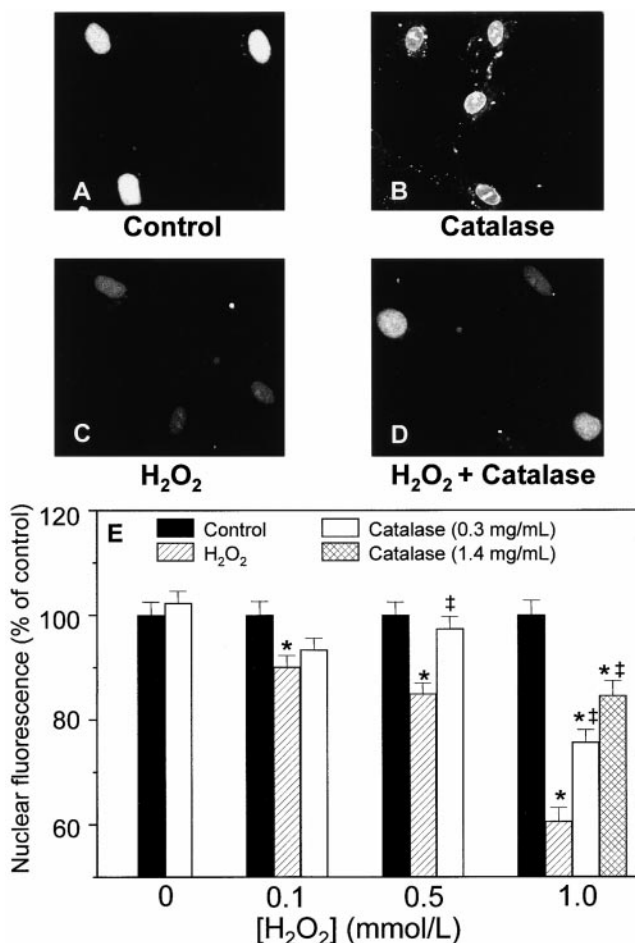

Figure 1. Nuclear protein import in aortic smooth muscle as a function of H_2O_2 treatment of the import cocktail. Import cocktail was untreated (A), treated with 0.3 mg/ml catalase (B), or treated with 1 mM H_2O_2 without (C) or with (D) 0.3 mg/ml catalase for 60 min at 37°C before import assay as described in Materials and Methods. Fluorescence was normalized relative to controls and plotted for various [H_2O_2] and [catalase] (E). Error bars represent SEM for 61 to 132 cells in three or four separate assays. *$P < 0.05$ vs. control. ‡$P < 0.05$ vs. H_2O_2-treated.

import activity. This decline was steeper when 1.0 mM H_2O_2 was used, compared with 0.1 mM. Experiments with very long-term exposures to H_2O_2 are difficult, since in aqueous solutions H_2O_2 eventually breaks down spontaneously to water and oxygen (Barnard and Matalon, 1992). H_2O_2 may also be scavenged by endogenous antioxidants. A blunting of the effect of H_2O_2 on import is eventually observed as a shallowing of the time-dependency curve (Fig. 2), most likely due to these phenomena.

In Fig. 3, the results of a 30-min pretreatment regimen with 1 mM H_2O_2 at 37°C are shown. Consistent with the results reported above, pretreatment of the import cocktail significantly reduced the measured nuclear fluorescence in treated versus control cells. However, when the permeablized cells themselves, rather than the import cocktail, were identically pretreated with H_2O_2, there was no effect, despite the higher [H_2O_2] used. Treatment of permeablized cells with 1 mM H_2O_2 for longer time peri-
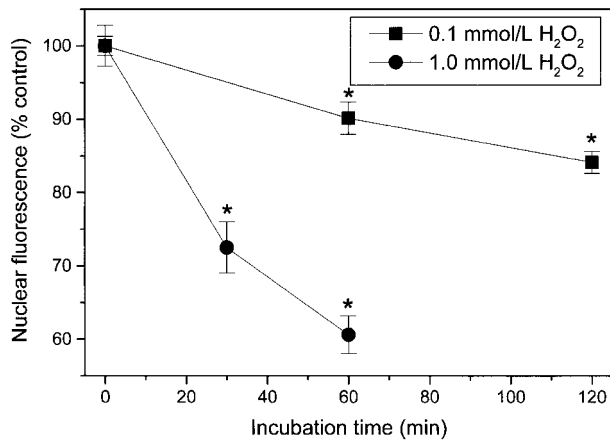
Figure 2. Nuclear import as a function of incubation time. Nuclear fluorescence was determined by confocal microscopy following import assay with control or H_2O_2 pretreated import cocktail for various times as described in Materials and Methods. Fluorescence was normalized relative to controls. Error bars represent SEM for 61 to 98 cells in three separate assays. $*P < 0.05$ vs. control.

ods than 30 min resulted in significant detachment from the coverslips and loss of cells during the import assay, making quantitation extremely difficult. The few remaining cells, however, appeared to accumulate import substrate in the nucleus at levels similar to controls (results not shown).

Studies of Oxidation by Free Radicals

The use of free radical generating systems to pretreat either import cocktail or permeablized cells resulted in different effects than those of H_2O_2 (Fig. 3). Superoxide radicals were generated by pretreatment of import cocktail with 2 mM xanthine plus 0.03 U/ml xanthine oxidase for 10
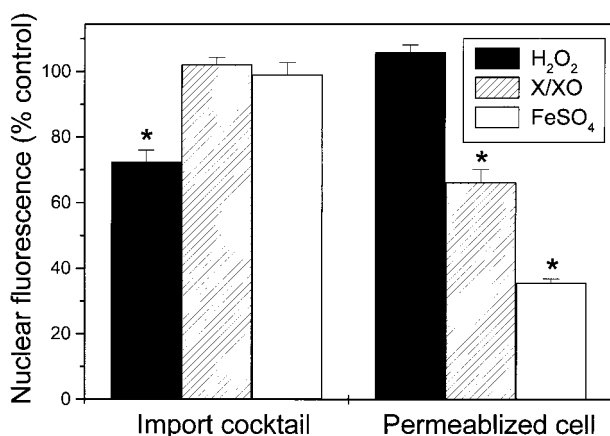

Figure 3. H_2O_2 acts on a cytosolic factor, unlike superoxide or hydroxyl radicals. Import cocktail or permeablized smooth muscle cells were treated with 1.0 mM H_2O_2 for 30 min, 2 mM xanthine plus 0.03 U/ml xanthine oxidase for 10 min (X/XO), or 0.1 mM H_2O_2 plus 0.1 mM $FeSO_4$ for 60 min at 37°C before import assay. Error bars represent SEM for 55 to 172 cells from three or four independent experiments. $*P < 0.05$ vs. control.

min at 37°C, but had no significant effect on nuclear protein import. Identical treatment of permeablized cells, however, caused a significant inhibition of import (~66% of control). Increasing treatment time of permeablized cells beyond 10 min caused significant detachment and loss of cells, making quantitation impossible. However, pretreatment of import cocktail with xanthine plus xanthine oxidase for times up to one hour showed no significant differences in import compared with controls (results not shown).

Since Fe^{2+} reacts with peroxide to generate hydroxyl radicals, import cocktail or permeablized cells were treated with 0.1 mM $FeSO_4$ plus 0.1 mM H_2O_2 for 60 min at 37°C. Similar results to those obtained with superoxide radicals were observed. Pretreatment of import cocktail caused no significant differences in import compared with controls, but pretreatment of permeablized cells significantly reduced import to ~36% of control values (Fig. 3).

ERK2 Activation Accompanies Import Inhibition by H_2O_2

The images in Fig. 4 show the results of nuclear import assay in control cells (Fig. 4 A) compared with results obtained when the import cocktail was treated for 60 min at 37°C with 1 mM H_2O_2 plus 45-min pretreatment with either 75 μM genistein (Fig. 4 C), a nonspecific tyrosine kinase inhibitor, or 75 μM daidzein, its inactive analogue (Fig. 4 D). Inclusion of 75 μM genistein alone had no effect on import (Fig. 4 B). Import in the cells in which the import cocktail pretreatment had included genistein resembled that in the controls, despite the presence of 1 mM H_2O_2. When the import cocktail contained H_2O_2 plus daidzein instead of genistein, import was significantly reduced to virtually the same degree as H_2O_2 alone.

These data are quantitatively represented in Fig. 4 E. The inclusion of 75 μM genistein in the import cocktail during the pretreatment with H_2O_2 caused a significant recovery of nuclear protein import (~86% of control) compared with pretreatment with H_2O_2 alone. This recovery was even greater than that observed by including 0.3 mg/ml catalase in the pretreatment protocol, and was quantitatively nearly identical to the recovery obtained by including 1.4 mg/ml catalase (Fig. 1 E). Inclusion of 75 μM daidzein failed to block the inhibition of import by 1 mM H_2O_2. Genistein or daidzein alone had no effect on import (Fig. 4 E).

Western blotting of control import cocktail was compared with pretreatment with 1 mM H_2O_2 ± 0.3 mg/ml catalase or 75 μM genistein, and immunostained with an anti-phospho-ERK2 antibody. Fig. 5 A presents a typical blot. The dark band in each lane represents phosphorylated ERK2 (p42), the activated form of ERK2. The band intensity is significantly increased when import cocktail is pretreated with 1 mM H_2O_2 for 60 min at 37°C compared with control. Conversely, inclusion of either 0.3 mg/ml catalase or 75 μM genistein during the pretreatment protocol is able to reverse this enhancement. The actual densitometric band intensity results for three experiments were quantitated and are shown in Fig. 5 B.

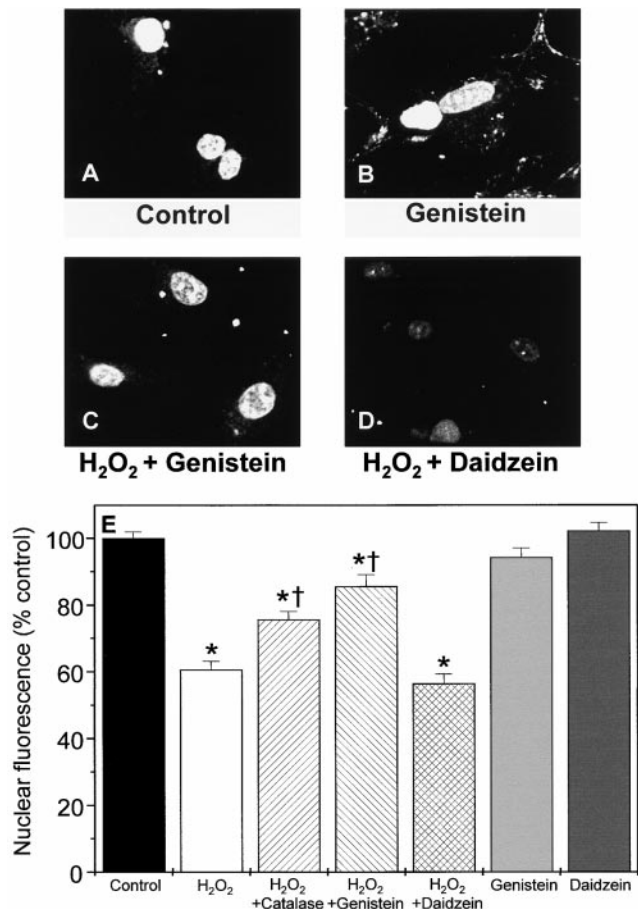To investigate whether ERK2 activation was coincidental with or causal for the alterations in import observed,

Figure 4. Inhibition of effects of H_2O_2 on nuclear protein import in aortic smooth muscle cells by genistein. Import cocktail was untreated (A), treated with 75 μM genistein (B), treated with 1 mM H_2O_2 plus 75 μM genistein (C), or with 1 mM H_2O_2 plus 75 μM daidzein (D) for 60 min at 37°C before import assay as described in Materials and Methods. Fluorescence was normalized relative to controls and plotted (E). Data representing treatment with H_2O_2 or H_2O_2 plus catalase is from Fig. 2. Error bars represent SEM for 78 to 112 cells in three or four separate assays. *$P < 0.05$ vs. control. †$P < 0.05$ vs. H_2O_2-treated.

import cocktail was pretreated with 20 μM PD98059, a specific inhibitor for the ERK2 activator MEK1, as described in Materials and Methods. The inhibition of nuclear protein import by 1 mM H_2O_2 was completely abolished by PD98059 (Fig. 6). Treatment of import cocktail with PD98059 alone had no effect on import (Fig. 6). Furthermore, treatment of import cocktail with 40 ng/ml activated ERK2 (i.e., phosphorylated ERK2) for 60 min at 37°C reduced nuclear import to ~57% of control (Fig. 6), similar to levels obtained with 1 mM H_2O_2 (Fig. 1 E). Identical treatment with 40 ng/ml nonactivated ERK2, or with 40 ng/ml activated ERK2 that had been inactivated by boiling for 20 min, had no effect on import (Fig. 6).

H_2O_2 Affects Ran/TC4 Localization

Localization of Ran/TC4 in rabbit aortic smooth muscle cells was examined by staining with anti-Ran/TC4 antibodies. Control cells exhibited high nuclear staining with
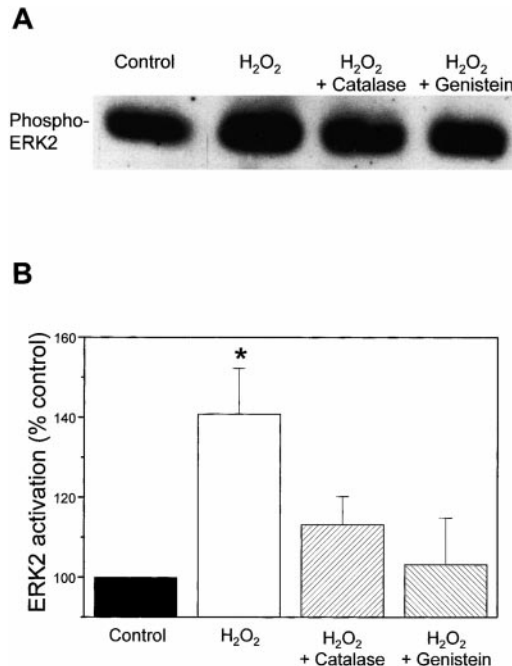

Figure 5. Activation of MAP kinase ERK2 in H_2O_2-treated import cocktail. Import cocktail was treated with 1 mM H_2O_2 for 60 min at 37°C, plus either 0.3 mg/ml catalase or 45-min pretreatment with 75 μM genistein. A, Shows representative bands obtained by Western blotting as described in Materials and Methods. B, The densitometric data obtained from the bands in A, normalized to controls. Error bars represent SEM for three separate experiments. *$P < 0.05$ vs. control.

low levels in the cytoplasm (Fig. 7, A–C). Upon treatment with 1 mM H_2O_2 for 60 min at 37°C, however, a distinct increase in cytoplasmic fluorescence is observed (Fig. 7, D–F). This rise in cytosolic Ran/TC4 could be attenuated by ei-
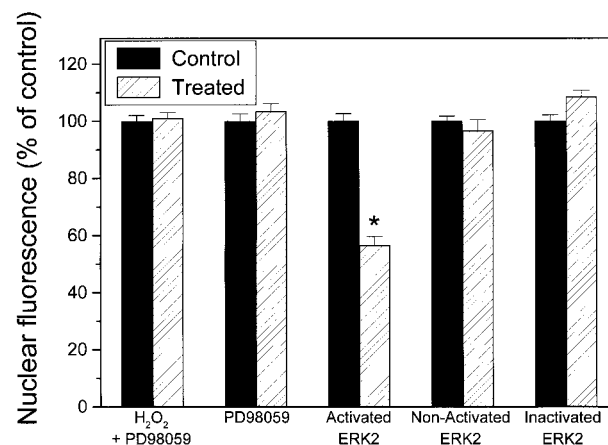

Figure 6. Modulation of ERK2 activity in import cocktail alters nuclear protein import. Import cocktail was untreated (control), or treated with 1 mM H_2O_2 plus 20 μM PD98059, 20 μM PD98059 alone, or 40 ng/ml activated ERK2 for 60 min at 37°C before import assay. Import cocktail was also treated for 60 min at 37°C with 40 ng/ml nonactivated ERK2 or 40 ng/ml activated ERK2 that had been boiled for 20 min and then used in import assays. Error bars represent SEM for 60 to 160 cells in three or four separate assays. *$P < 0.05$ vs. control.
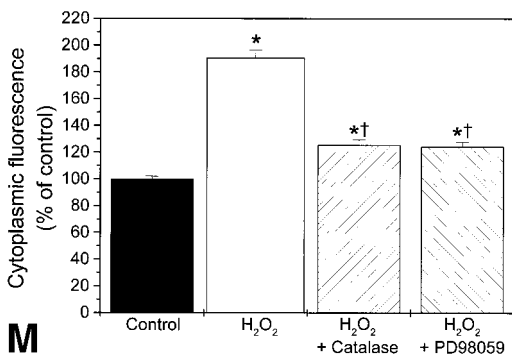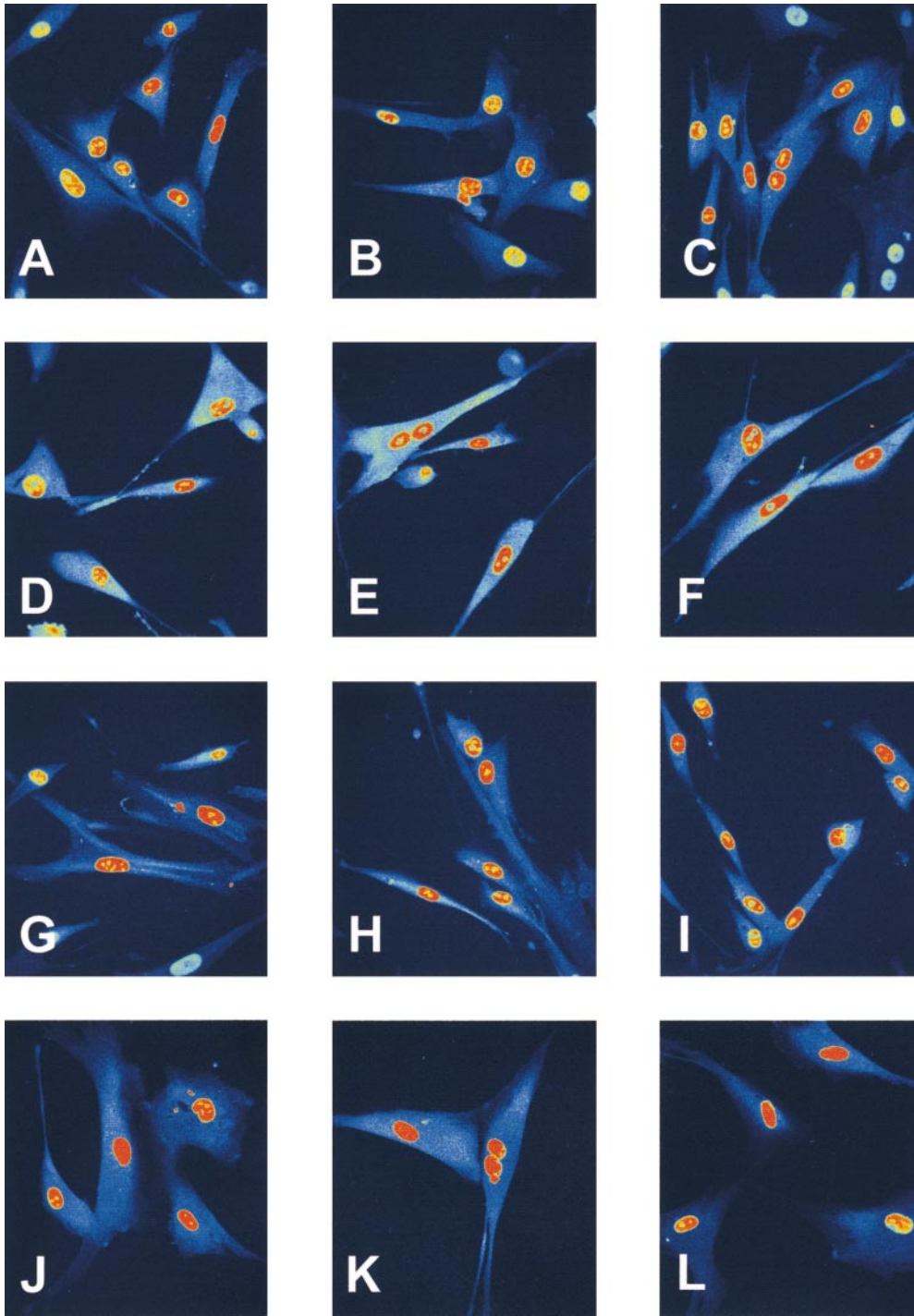
Figure 7. H_2O_2 causes Ran/TC4 translocation to the cytosol. Rabbit aortic smooth muscle cells were treated with 1 mM H_2O_2 with or without 0.3 mg/ml catalase or 20 μM PD98059 as described in Materials and Methods, and then were visualized by anti-Ran/TC4 immunolabeling. A–C, Control cells; D–F, H_2O_2-treated cells; G–I, treated with catalase and H_2O_2; and J–L, treated with PD98059 and H_2O_2. These figures are representative of three separate experiments. M is the quantitative analysis of A–L. Error bars represent SEM for three separate experiments. *$P < 0.05$ vs. control. †$P < 0.05$ vs. H_2O_2-treated.

ther 0.3 mg/ml catalase (Fig. 7, G–I) or 20 μM PD98059 (Fig. 7, J–L). Quantitation of fluorescence levels reveals that H_2O_2 treatment nearly doubled the Ran signal in the cytoplasm (Fig. 7 M). Whereas catalase and PD98059 were unable to completely restore control levels of cytoplasmic Ran, they did cause a significant decrease in cytoplasmic Ran compared with peroxide-treated cells.

Discussion

This is the first report that nuclear protein import in aortic vascular smooth muscle cells is inhibited by H_2O_2. Theoretically, H_2O_2 may alter nucleocytoplasmic transport through an effect on a cytoplasmic factor or an action on the nuclear pore complex itself. To determine whether H_2O_2 could exert its effects on import by attacking the nuclear pore complex or nuclear membrane, permeablized smooth muscle cells were pretreated with 1 mM H_2O_2 before import assay. No effect was observed when the permeablized cells were treated with H_2O_2 (Fig. 3). However, the same concentration of H_2O_2 resulted in a large decrease in import when the import cocktail was treated (Fig. 1 E). These data suggest that H_2O_2 can affect cytosolic factors found in the import cocktail, but not the pore complex itself.

One potential mechanism for the action of H_2O_2 on import is that it may cause oxidation of cytosolic factors required for import (e.g., the NLS receptor or other components of the import complex). To investigate this possibility, two free radical generating systems were used to treat either import cocktail or permeablized cells used in the import assay. Xanthine plus xanthine oxidase produces superoxide radicals, while free iron reacts quickly with H_2O_2 to produce hydroxyl radicals (Burton et al., 1984; Kloner et al., 1989; Kaul et al., 1993). In contrast to the findings with H_2O_2, pretreatment of the import cocktail with either free radical generating system had no effect on import (Fig. 3). Increasing the time of pretreatment with xanthine/xanthine oxidase to one hour still had no effect on import (results not shown). Conversely, when permeablized cells were pretreated with either free radical generating system, import was significantly inhibited (Fig. 3). Together, these data reveal that H_2O_2 exerts its effect on import using a completely different mechanism than superoxide or hydroxyl radicals, and suggest that oxidation is not involved. They also demonstrate that oxidation of cell structural components, including the nuclear pore complex, can significantly depress nuclear protein import.

H_2O_2 has been shown to activate the MAP kinase ERK2 in vascular smooth muscle (Cantoni et al., 1996; Guyton et al., 1996; Rao, 1996; Li et al., 1997) and cardiomyocytes (Sabri et al., 1998). It can also activate other signal transduction pathways, such as the JNK pathway (Lo et al., 1996). We investigated whether H_2O_2-induced ERK2 activation may play a role in the H_2O_2-induced inhibition of import. When import cocktail was treated before H_2O_2 exposure with genistein, an inhibitor of tyrosine kinase-mediated phosphorylation, import was attenuated to levels similar to those obtained with catalase treatment (Fig. 4). Daidzein, the inactive analogue of genistein, exhibited no effect. Genistein and daidzein had no effect on import on their own. H_2O_2 activated ERK2 as shown by Western

blotting, an effect which could be reversed by catalase or genistein (Fig. 5). Together, these data implicate ERK2 as a mediator of nuclear protein import inhibition by H_2O_2. These results also explain why treatment of permeablized cells with H_2O_2 did not affect import, since the H_2O_2 would have been washed away before the import cocktail was applied to complete the assay, and therefore would be unavailable to affect ERK2.

To further investigate the role of ERK2 in inhibition of import, import cocktail was also pretreated with 20 μM PD98059 before H_2O_2 treatment. This compound blocks MEK1, a protein kinase immediately upstream to ERK2 in the MAP kinase signal cascade and a direct activator of ERK2. Like genistein, PD98059 was able to reverse the effects of H_2O_2 on import. However, unlike genistein, PD98059 completely normalized import to control values (Fig. 6). It therefore appears that, while ERK2 does mediate the effect of H_2O_2 in inhibiting import, H_2O_2 exerts its primary effect further up the signal cascade. If H_2O_2 activated ERK2 directly, PD98059 would not be expected to have an effect on reversing H_2O_2-induced inhibition of import. Rao has shown that H_2O_2 treatment of intact vascular smooth muscle cells induces SHC-Grb2-SOS complex formation with EGF-receptor tyrosine kinase (Rao, 1996). This process leads to activation of Ras, which presumably would lead to activation of the entire MAP kinase cascade leading to ERK2, which was also observed to be activated in Rao's study. It is likely that in our experiments, a similar process is occurring. However, since the import cocktail is devoid of plasma membrane, the involvement of receptor tyrosine kinases is unlikely. H_2O_2 must be exerting its effect, therefore, on another stage of the MAP kinase signaling cascade or on cytosolic tyrosine kinases, although the exact primary target remains unidentified. Clerk et al. (1998) have suggested the possibility that H_2O_2 may inactivate a phosphatase higher up in the ERK2 signaling pathway, resulting in activation of MAP kinases.

Further evidence to support the role of ERK2 is shown in Fig. 6. Treating import cocktail with an activated form of ERK2 alone results in a significant inhibition of nuclear protein import. This effect is similar in magnitude to the inhibition of import observed by treatment with the highest concentrations of H_2O_2 used in this study. Nonactivated ERK2 or activated ERK2 that has been inactivated by boiling, do not inhibit import (Fig. 6). Combined with the results discussed above, it appears that the level of activation of ERK2 determines inhibition of nuclear protein import.

The ultimate downstream target of ERK2 activation that affects nuclear protein import is unclear. Phosphorylation of the import substrate is not responsible, since the custom NLS used in this study lacks upstream phosphorylation sites. The nuclear pore complex is also not responsible, since H_2O_2 treatment of permeablized cells had no effect on import (Fig. 3). The most likely target is a cytosolic import factor, as discussed earlier. Ran/TC4 is a Ras-related GTPase required for protein import that cycles between the nucleus and cytoplasm (Avis and Clarke, 1996; Melchior and Gerace, 1998). When Ran/TC4 localization was examined by immunocytochemistry, it was found that H_2O_2 nearly doubled the cytosolic level of Ran (Fig. 7). This effect could be significantly attenuated by treatment

with either catalase or PD98059, suggesting that this shift was due to ERK2 activation by H_2O_2. Although GTP hydrolysis by Ran is not required for import to occur, it does appear to be required for proper cycling of Ran between the nucleus and the cytoplasm (Schwoebel et al., 1998). Disrupting Ran cycling between these compartments by altering GTP hydrolysis by Ran inhibits nuclear protein import (Schlenstedt et al., 1995; Avis and Clarke, 1996; Schwoebel et al., 1998). The changes we observed in Ran localization suggest that ERK2 activation may alter Ran cycling, although the exact mechanism of this process remains unknown. Possibilities include alterations in Ran GTPase activity, resulting in an increase in cytosolic Ran-GTP, which destabilizes the import complex and inhibits import (Schlenstedt et al., 1995; Avis and Clarke, 1996), or an inhibition of RanGAP1, the Ran GTPase activating protein, which would also increase cytosolic Ran-GTP. It is also possible, however, that other import factors are involved, either currently identified or still unknown.

In both physiological and pathological situations, there may be constant, long-term generation of H_2O_2. Our results demonstrate that even low concentrations of H_2O_2 acting over longer periods of time can progressively inhibit import (Fig. 2). This finding is of particular importance in the atherogenic vascular intima, in which infiltrating macrophages and monocytes may be a constant source of H_2O_2 production (Parthasarathy et al., 1986). Since H_2O_2 readily crosses cell membranes (Halliwell and Gutteridge, 1990), it is free to move throughout the intima and affect cells distal to the site of H_2O_2 production. The half-life of H_2O_2 in complex aqueous solutions can be quite short (on the order of minutes; Barnard and Matalon, 1992; Hyslop et al., 1995) due to spontaneous breakdown and endogenous scavenging. Both are relevant under our experimental conditions. We used higher doses of H_2O_2 (1 mM) over the course of one-hour exposures for some of the experiments. This approach appears justified, since exogenously added ERK2 closely mimicked the highest concentration of H_2O_2 used in this study with regard to import inhibition (Fig. 6). The LD_{50} for H_2O_2 in PC12 cells is reported as 350 μM, and at 750 μM H_2O_2 there is still significant cell survival (Guyton et al., 1996). These levels are similar to those used in our study. Moreover, since permeablized cells or cell cytosol was used throughout the study, toxicity was not an issue.

Our results have important physiological and pathological significance. Physiologically, it has been suggested that H_2O_2 is required for signal transduction in response to PDGF or angiotensin II in vascular smooth muscle (Sundaresan et al., 1995; Zafari et al., 1998). To mimic the effects of PDGF, 0.1–1.0 mM exogenous H_2O_2 was required, the same range as that used in this study (Sundaresan et al., 1995). It also recently has been suggested that H_2O_2 may itself be a second messenger, similar to the role played by nitric oxide (Griendling and Harrison, 1999). This role would be entirely consistent with the findings reported in the present study. The respiratory burst of monocytes and macrophages during microbial killing releases significant amounts of activated oxygen species, including H_2O_2 (Babior, 1978). Activated oxygen species are also produced as the result of the normal leakage of electrons out of mitochondrial electron transport chains (Boveris, 1977).

Pathologically, H_2O_2 is generated in cells during ischemia (Burton et al., 1984; Kloner et al., 1989; Bast et al., 1991; Kaul et al., 1993; Vanden Hoek et al., 1997). Hyslop et al. (1995) have reported striatal [H_2O_2] well above 150 μM during global forebrain ischemia in the rat. Activated oxygen species including H_2O_2 also play a significant role in atherogenesis by stimulating oxidation of lipoproteins and proliferation of smooth muscle cells. These species are produced by macrophages and monocytes infiltrating the vascular intima, as well as by endothelial and smooth muscle cells themselves (Morel et al., 1984; Parthasarathy et al., 1986).

The actual effects of H_2O_2 on smooth muscle are somewhat controversial. H_2O_2 has been demonstrated to cause both smooth muscle proliferation and apoptosis (Abe et al., 1994; Cantoni et al., 1996; Li et al., 1997; Zafari et al., 1998), and in both situations is associated with DNA synthesis and ERK2 activation (Fiorani et al., 1995; Cantoni et al., 1996; Li et al., 1997). Recent evidence supports the hypothesis that H_2O_2 may be a key modulator of smooth muscle cell survival and proliferation (Brown et al., 1999). The critical event that determines whether the cell proliferates or dies after H_2O_2 exposure is unknown, but has been hypothesized to depend on whether exposure is acute or tonic, or how long ERK2 remains activated (Tombes et al., 1998). ERK2, on activation, translocates to the nucleus via an NLS-independent pathway (Fukuda et al., 1997; Yu et al., 1999). In the nucleus, ERK2 activates mRNA transcription of a number of transcription factors, including c-*fos*, c-*jun*, and *fra-1* (Guyton et al., 1996; Li et al., 1997; Zhang et al., 1998). Our data show that ERK2 inhibits NLS-dependent nuclear protein import. This may explain how smooth muscle becomes apoptotic concomitant with activation of ERK2: critical growth signals may be prevented from entering the nucleus due to import inhibition, while ERK2 is still able to gain entry to the nucleus via the alternate pathway. This hypothesis is also consistent with the findings that reduction of nuclear import is associated with cell quiescence (Feldherr and Akin, 1991, 1993). Our data are not consistent with the pro-growth effects of H_2O_2, although we cannot rule out the possibility that import inhibition prevents growth repressors from entering the nucleus and inhibiting transcription of critical genes. Further work in these areas is necessary.

In summary, this is the first demonstration that activated ERK2 is able to inhibit NLS-dependent nuclear protein import. H_2O_2 inhibits nuclear protein import in a dose- and time-dependent manner by activation of ERK2. Similar exposure of intact cells to H_2O_2 alters the distribution of Ran/TC4 between the nucleus and cytosol. Superoxide and hydroxyl radicals are able to inhibit nuclear import by interaction with nuclear structures, but do not mimic the effects of H_2O_2. The data are particularly significant in situations where H_2O_2 is produced at high levels or over long periods of time, such as during ischemia or atherogenesis. The finding that ERK2 activation affects nuclear protein import has implications for studies of cell proliferation, angiogenesis, hypertrophy, and diseases such as cancer and diabetes.

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