Redox-dependent Regulation of Nuclear Import of the Glucocorticoid Receptor*

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A number of transcription factors including the glucocorticoid receptor (GR) are regulated in a redox-dependent fashion. We have previously reported that the functional activity of the GR is suppressed under oxidative conditions and restored in the presence of reducing reagents. In the present study, we have used a chimeric human GR fused to the Aequorea green fluorescent protein and demonstrated that both ligand-dependent and independent nuclear translocation of the GR is impaired under oxidative conditions in living cells. Substitution of Cys-481 for Ser within NL1 of the human GR resulted in reduction of sensitivity to oxidative treatment, strongly indicating that Cys-481 is one of the target amino acids for redox regulation of the receptor. Taken together, we may conclude that redox-dependent regulation of nuclear translocation of the GR constitutes an important mechanism for modulation of glucocorticoid-dependent signal transduction.

Glucocorticoids are indispensable not only for maintenance of metabolic homeostasis but also for treatment of a wide variety of human disorders including inflammatory diseases (1-4). Glucocorticoids exert hormone action in target tissues via binding to the glucocorticoid receptor (GR). The GR, as a ligand-inducible transcription factor belonging to the nuclear receptor superfamily, is docked in the cytoplasm in the absence of hormonal ligands. Upon hormone binding, the GR dissociates from hsp90 and translocates into the nucleus to regulate target gene expression (5-8). Previous biochemical studies have proven that GR function is sensitive to redox state in vitro, most possibly via reversible modification of cysteine residues in the GR. For instance, oxidative modification of the GR decreases ligand binding and nonspecific DNA binding activities of the GR in vivo (9-13). Moreover, we have recently presented evidence demonstrating that glucocorticoid hormone action in vivo is strictly controlled by cellular redox state and cysteine-affinitive metal ions (14-19). Although a number of transcription factors have been shown to be regulated in a redox-dependent fashion (reviewed in Refs. 20-22), the molecular mechanism for redox regulation of cellular GR remains largely unknown.

From a signal transduction point of view, the glucocorticoid signal that finally influences gene expression must be transmitted to the nucleus via receptor translocation. Therefore, nuclear import of the GR is one of the key control points in regulation of glucocorticoid hormone action. In general, protein transport from the cytoplasm to the nucleus involves the nuclear localization signal (NLS), i.e. short peptide sequences that are necessary and sufficient for nuclear localization of their respective proteins (23). One of the best characterized NLS motifs is that of SV40 large tumor antigen (T-ag) (23). Nuclear import of the GR is mediated by NL1, a stretch of basic amino acids at the immediate C-terminal end of the receptor DNA binding domain, and a second significantly less characterized NLS in the ligand binding domain, NL2 (24). Whereas the NLS of SV40 T-ag consists of a short domain of basic amino acids, NL1 of the GR is a bipartite domain and confers constitutive nuclear localization of the receptor (24). In contrast, NL2 acts as a dominant negative NLS in the absence of ligands (24). A number of studies have proven that the GR shuttles between the cytoplasm and the nucleus, and subcellular localization of the GR is determined by an equilibrium of both nuclear import and export. The GR translocates to the nucleus in a ligand- and energy-dependent manner, and nuclear export of the GR also requires ATP (25-34). On the other hand, subcellular localization of certain transcription factors is conditionally regulated to confer extracellular stimulus-dependent gene expression. For example, cellular treatment with tumor growth factor-β causes nuclear translocation of the transcription factors Smad3/Smad4 (35). Moreover, oxygen also variably modulates intracellular compartmentalization of several transcription factors including nuclear factor-κB (36). In the case of the GR, it is well known that glucocorticoid ligands are a unique molecular switch to trigger nuclear translocation of the receptor; however, it has not yet been documented whether the cellular redox state modulates subcellular localization of the receptor.

In the present study, we have used a fusion protein of human GR and Aequorea green fluorescent protein (GFP) to study nuclear translocation of the GR in living cells (26). Using this
model system, we demonstrate that not only ligand-dependent but also ligand-independent nuclear import of the GR is negatively modulated under oxidative conditions, illustrating the critical importance of the cellular redox state in modulation of GR-mediated signal transduction.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Dexamethasone was purchased from Sigma, and other chemicals were from Wako Pure Chemical (Osaka, Japan) unless otherwise specified.

For the construction of the expression plasmid for the chimeric protein of GFP and the human GR, pGFP-hGR, was described previously (referred to as pCMX-GFP-hGR in Ref. 26). The construction of pCMX-GFP-VP16-GR DBD, which encodes fusion protein for GFP, VP16 transactivation domain, and the DNA binding domain (DBD) of the human GR (serine 403 to leucine 532), is described elsewhere (15). pGFP-hGR/C481S, which carries a TGT to TCT point mutation to generate a cysteine for serine substitution at position 481 in the human GR, was constructed using pGFP-hGR as a template with the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The oligonucleotides used were 5’-GCGCTATCCAAGAACAATCTTGACGCCATAGAATCG-3’ and 5’-GGGTTTCATTCCAGCCTGAAGAGATTTTCGATAGCGGCATGC-3’. pCMX-GFP-C481S was confirmed by DNA sequencing. The glucocorticoid-responsive reporter plasmid pGRE-Luc has been described elsewhere, in which the firefly luciferase gene expression is driven under the control of a tandem repeat of glucocorticoid response elements (17). The β-galactosidase expression plasmid pCH110 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as an internal control for transfection efficiency when appropriate.

**Cell Culture—**The human GR-expressing CHO cells, CHOpMTGR, were originally developed and kindly provided by Dr. Stefan Nilsson (Karol Bio, Huddinge, Sweden) and cultured in Ham’s F-12 medium (Life Technologies, Inc.) (37). COST cells and HeLa cells were obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan) and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Iwaki Glass Inc., Chiba, Japan). CHO-K1 cells were obtained from the RIKEN Cell Bank and maintained in Ham’s F-12 medium. All media used in this study were phenol red-free and supplemented with 10% fetal calf serum (FCS) and antibiotics. Serum steroids were stripped with dextran-coated charcoal (DCC), and cells were cultured in a humidified atmosphere of 5% CO2, unless otherwise specified.

**Immunocytochemical Analysis of Subcellular Localization of the GR—**Cells grown on eight-chambered sterile glass slides (Nippon Beco Ltd., Tokyo, Japan) were fixed for immunostaining using a freshly prepared solution of 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS) overnight at 4°C. Immunocytochemistry was carried out as described previously (38) with a small modification. Briefly, cells were fixed for five times with 0.1% Triton X-100 for 1 h at room temperature, and then the cells were washed five times with PBS and incubated with fluorescein isothiocyanate-conjugated streptavidin at a dilution of 1:100 in PBS containing 0.1% Triton X-100 for 1 h at room temperature. Finally, the cells were washed five times with PBS and mounted with GEL/MOUNT™ (Biomed Co. Ltd., Foster city, CA) and then examined by a Zeiss Axiosvert 135 microscope equipped with a fluorescein isothiocyanate filter set.

**Transfection and Reporter Gene Assay—**Before transfection, cell culture medium was replaced with Opti-MEM medium lacking phenol red (Life Technologies). A plasmid mixture containing pGRE-Luc in the presence or absence of pGFP-hGR expression plasmid was mixed with TransIT-LT1 reagent (Panvera Corp., Madison, WI) and added to the culture. The total amount of the plasmids was kept constant, adding an irrelevant plasmid (pGEM3Z) was used unless otherwise specified. After 6 h of incubation, the medium was replaced with fresh DMEM supplemented with 2% DCC-treated FCS, and the cells were further cultured in the presence or absence of various ligands for 24 h at 30°C to increase transactivation function of expressed GFP-hGR (26). Luciferase enzyme activity was determined using a luminometer (Berthold GmbH & Co. KG, Bad Wildbad, Germany) essentially as described before (17).

**Visualization of Intracellular Trafficking of GFP Fusion Proteins in Living Cells—**For analysis of nuclear translocation of the GR in living cells, we transiently expressed GFP-tagged human GR or its mutants in COS7 cells. The cells were cultured on the silane-coated coverslips in 6-cm diameter glass dishes in DMEM at the medium was changed with Opti-MEM medium lacking phenol red before transfection. A plasmid mixture containing 6 µg of expression plasmids for various GFP-tagged proteins was mixed with 12 µl of TransIT-LT1 reagent and added to the culture. After 6 h of incubation, the medium was replaced with DMEM supplemented with 2% DCC-treated FCS, and the cells were cultured at 30°C for at least 4 h, and then at 37°C thereafter. GFP was expressed at detectable levels between 24 and 72 h after transfection. Routinely, cells were used for further experiments 48 h after transfection. After various treatments, cells were examined using a Zeiss Axiosvert 135 microscope enclosed by an incubator and equipped with a heating stage, a fluorescent isothiocyanate filter set, and epifluorescence with illumination from a Giken burner (26). Quantitative assessment of the subcellular localization of expressed GFP fusion proteins was performed according to methods described elsewhere (39). In brief, subcellular localization analysis of GFP-tagged proteins was performed by blinded observers, counting approximately 200 cells in which GFP fluorescence was detected. The GFP fluorescence-positive cells were classified into four different categories: N < C for cytoplasmic fluorescence; N + C for having equal fluorescence in the cytoplasmic and nuclear compartments; N > C for nuclear-dominant fluorescence; and N for exclusive nuclear fluorescence.

**Immunoprecipitation and Western Immunoblot Assays—**Whole cell extract was prepared by lysing cells treated with dexamethasone and/or H2O2 in 25 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 8.2), 1 mM EDTA, 50 mM NaCl, 2.5 mM molybdate, and 10% glycerol. Immunoprecipitation experiments, with either the anti-hsp90 IgM antibody 3G3 (Affinity Bioreagents) or control mouse IgM antibody TEPC 183 (Sigma) was carried out as described previously (40). Briefly, goat anti-mouse IgM (Sigma) was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) by incubating in the coupling buffer (0.1 mM NaHCO3, 1% NaCl, pH 8.3) overnight at 4°C. Thirty-five micrograms of either the monoclonal anti-hsp90 IgM antibody or control mouse IgM antibody was then incubated with 80 µl of a 1:1 suspension of the goat anti-mouse IgM antibody coupled to Sepharose in MENg buffer (25 mM Mops (pH 7.5), 1 mM EDTA, 0.02% NaN3, 10% glycerol) on ice for 90 min. This Sepharose-adsorbed material was then pelleted and washed successively once with 1 ml of MENg buffer containing 0.5 mM NaCl and twice with MENg buffer containing 20 mM sodium phosphate buffer. After brief centrifugation, the pellet was resuspended in 80 µl of MENG buffer containing 20 mM sodium molybdate, 2 mM diethiothreitol, 0.25 mM NaCl, and 2.5% (w/v) bovine serum albumin. In immunoprecipitation experiments, 66 µg of cellular protein was added to the resuspension. The reaction mixtures were incubated on ice for 90 min, after which Sepharose beads were pelleted by centrifugation and washed three times with MENg buffer containing 20 mM sodium phosphate buffer. Immunoblots were performed in both OCT-methanol and acetone-ethanol mixture and then transferred to a nitrocellulose filter. The immunoblots were visualized using the enhanced chemiluminescence method according to the manufacturer’s protocol (Amer- sham Pharmacia Biotech). After autoradiography, intensities of the appropriate bands for GFP-hGR and hsp90 were quantified using a densitometer.

**Protein Glass and Microinjection of Recombinant GST-NLS-GFP Proteins—**Recombinant GST-NLS-GFP, encoding the NLS of SV40 T-ag, glutathione S-transferase (GST), and GFP, was prepared as described previously (41). Glass capillary-mediated transfer of the recombinant protein was carried out using a Leitz micromanipulator as also described previously (41). In brief, COST cells were plated on the silane-coated coverslips in 35-mm diameter dishes in DMEM supplemented with 2% DCC-stripped FCS and treated with or without 2 mM H2O2 for
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Inhibition of Hormone-dependent Nuclear Import of the GR by Treatment with H₂O₂—We recently reported that the functional activities of the GR, including ligand binding, DNA binding, and transactivation, are strictly controlled by the cellular redox state. Since, as described in the Introduction, nuclear translocation is a prerequisite for the GR to mediate hormone signaling, we wanted to examine whether subcellular localization of the GR is modulated via redox-dependent mechanisms. For this purpose, first, we assessed whether treatment with H₂O₂ influenced subcellular localization of the GR by indirect immunofluorescence analysis using human GR-expressing CHO-pMTGR cells. As shown in Fig. 1, we found that ligand-dependent nuclear targeting of the human GR is markedly inhibited after treatment with 1 mM H₂O₂, whereas pretreatment with N-acetyl-L-cysteine (NAC) effectively titrates this negative effect of H₂O₂. Based on these results, we decided to transiently express GFP-hGR chimeric protein (26) to perform a kinetic analysis of subcellular localization of the GR in living cells with special reference to the regulatory role of cellular redox state. In these experiments, we also examined whether the nuclear translocation process was individually controlled by a redox-dependent mechanism.

To this end, we tested whether GFP-hGR mimics endogenous GR in terms of ligand-dependent nuclear translocation and transactivation using COS7 cells, since those cells show relatively high transfection efficiency. After transient transfection of GFP-hGR into COS7 cells, 20–30% of the cells showed cytoplasmic green fluorescence, indicating expression of GFP-hGR fusion protein in those cells. In the absence of hormone, expressed GFP-hGR chimera localized exclusively in the cytoplasm, whereas it translocated into the nucleus in a timedependent manner after hormone treatment (Fig. 2, and data not shown). Quantitative analysis (see “Experimental Procedures”) also revealed a strict ligand concentration dependence of nuclear translocation of GFP-hGR (Fig. 2). This hormone-dependent nuclear localization of GFP-hGR correlated with reporter gene expression (data not shown). We also performed similar experiments using CHO-K1 cells and HeLa cells and found that, despite significantly lower transfection efficiency, GFP-hGR again mimics native GR in these cells as well (data not shown). Therefore, although GFP was present in the N-terminal end of the human GR, we concluded that GFP-hGR mimics the native human GR, at least with reference to ligand-dependent translocation and transactivation properties (26, 28, 33).

We next examined the effect of oxidative treatment on nu-
clear translocation of GFP-hGR in COS7 cells. As shown in Fig.
3, the rate of ligand-dependent nuclear translocation of GFP-
hGR was markedly delayed upon the addition of H₂O₂. This
suppressive effect of H₂O₂ was dose-dependent (Fig. 3). Indeed,
in the presence of 2 mM H₂O₂, nuclear translocation was se-
verely compromised even after treatment with 100 nM dexa-
methasone (Fig. 3). Neither distribution nor intensity of GFP
fluorescence was significantly influenced after treatment with
H₂O₂ when GFP alone was expressed in COS7 cells (data not
shown and Fig. 5). To test the reversibility of H₂O₂-dependent
suppression of nuclear translocation, GFP-hGR-expressing
COS7 cells were treated with 10 mM NAC after 1-h treatment
with 2 mM H₂O₂, and H₂O₂ was completely reconstituted in the
presence of 100 nM dexamethasone. As shown in Fig.
3, NAC partially but efficiently reversed the negative effects of
2 mM H₂O₂, resulting in the nuclear translocation of GFP-hGR
in the presence of 100 nM dexamethasone. These results
strongly indicate that the suppressive effects of H₂O₂ can be
inhibited by the addition of reducing reagents and do not ap-
pear to be related to a decrease in cell viability.

We also compared the sensitivity to H₂O₂ of nuclear trans-
location of GFP-hGR in COS7 cells, CHO-K1, and HeLa cells,
all of which revealed almost complete nuclear translocation of
GFP-hGR after treatment with 100 nM dexamethasone in 2 h
data not shown). When concentration-dependent curves were
compared, the different cells showed the following rank order of
sensitivity to oxidative treatment: HeLa, CHO-K1, and COS7
cells. IC₅₀ values for inhibition of nuclear translocation of GFP-
hGR were 0.49, 0.78, and 1.00 mM for HeLa, CHO-K1, and
COS7 cells, respectively. These data indicate that, despite var-
ation in sensitivity to oxidative treatment, oxidative inhibition
of nuclear translocation of the GR may be a general phenomenon.

Oxidative Stress Inhibits Ligand-dependent Dissociation of
hsp90 from the GR—
To assess the mechanism of oxidation-
mediated repression of GR nuclear translocation, we investi-
gated whether treatment with H₂O₂ influenced the ligand-de-
dpendent dissociation of hsp90 from the GR, since we
previously have reported that oxidative treatment decreases
ligand binding activity of the GR (16, 17) and ligand-dependent
dissociation of hsp90 has been demonstrated to correlate with
initiation of nuclear translocation (29). First, to demonstrate
interaction between the GR and hsp90, we employed in coimmunoprecipitation experiments a monoclonal IgM antibody capable of recognizing both free hsp90 and hsp90 complexed with other proteins (40). These experiments revealed that GFP-hGR coprecipitated with hsp90 using whole cell extracts from pGFP-hGR-transfected COS7 cells (Fig. 4A). In contrast, only background levels of GFP-hGR were coprecipitated by control IgM antibodies (Fig. 4A), indicating that GFP-hGR as well as native human GR forms a stable complex with hsp90 in solution in the absence of ligands. The IgM anti-hsp90 antibody did not react with GFP itself (data not shown). We then examined the effect of H2O2 on GFP-hGR-hsp90 interaction. After transfection of pGFP-hGR to COS7 cells, cells were cultured in the absence or presence of 1 and 2 mM H2O2 for 2 h, exposed to 100 nM dexamethasone for 30 min and harvested, and then whole cell extracts were prepared. Fig. 4B clearly demonstrates that disassociation of hsp90 from GFP-hGR requires ligand (top part, compare lanes 2 and 3). Moreover, treatment with H2O2 appeared to partially suppress this ligand-dependent dissociation of hsp90. The intensities of the bands in lanes 4 and 5 were 22 and 48% of that of lane 2 in Fig. 4B, respectively, when analyzed densitometrically (top part). On the other hand, treatment with H2O2 did not significantly affect complex formation between GFP-hGR and hsp90 in the absence of ligand (data not shown). Western immunoblot analysis of whole cell extracts using either the anti-GFP or anti-hsp90 antibodies revealed that total amounts of expressed GFP-hGR and cellular hsp90 were not significantly affected under these experimental conditions (Fig. 4B, middle and bottom). Thus, these data suggest that oxidative treatment, most possibly via interference with ligand binding, suppresses subsequent dissociation of hsp90 from the receptor. However, note that oxidative inhibition of ligand-dependent dissociation of hsp90 is partial.

**Hormone-independent Nuclear Translocation Is Also Negatively Modulated under Oxidative Conditions**—The segregation between the effects of oxidative treatment on hsp90 release from the GR and those on nuclear translocation of GR prompted us to consider that the effects of oxidative treatment on these two phenomena are separable, although both of these events occur subsequent to ligand binding. It has already been shown that the truncation of the ligand binding domain of the GR results in constitutive nuclear localization of the mutant receptor (41). Therefore, to eliminate the involvement of the effect on ligand-receptor interaction, we constructed the expression plasmid for the fusion protein of GFP, VP16 transactivation domain, and the DNA binding domain of the GR (15). We have already shown that this plasmid contains NL1 of the GR and constitutively localizes in the nucleus (15). After transient expression of GFP-VP16-GR DBD in COS7 cells, the cells were cultured in the presence or absence of H2O2 for 12 h, and subcellular localization of this fusion protein was analyzed using a fluorescent microscope. In the absence of H2O2, GFP-VP16-GR DBD was constitutively localized in the nucleus (Fig. 5). However, in the presence of 1 mM H2O2—part of the cells showed cytoplasmic retention of GFP fluorescence (Fig. 5). Moreover, almost all cells having GFP fluorescence revealed significant cytoplasmic fluorescent signal at 2 mM H2O2 indicating that the expressed fusion protein between GFP, VP16, and GR DBD, at least in part, docks in the cytoplasm under oxidative conditions (Fig. 5). We thus may conclude that the GR nuclear translocation process is negatively modulated in a redox-

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**Fig. 6.** Effect of oxidative treatment on nuclear export of the GR. GFP-hGR-expressing COS7 cells were treated with 100 nM dexamethasone (Dex) for 1 h to allow cells to show almost complete nuclear translocation of GFP-hGR. After withdrawal of hormone by intensive washing with PBS three times, the medium was replaced with OptiMEM medium lacking phenol red. Then the cells were incubated in the absence (open circles) or presence of various concentrations of H2O2 (0.5 mM [open triangles], 1 mM [open squares], 2 mM [filled squares]), and subcellular localization of GFP-hGR was assessed at indicated time points. Filled circles represent the percentages of the cells belonging to category N when the cells were further cultured in the presence of 100 nM dexamethasone. Quantitative analysis was performed as described under “Experimental Procedures.” Experiments were repeated three times with almost identical results, and a representative graph is shown.

**Fig. 7.** Nuclear translocation of GST-SV40 T-ag NLsc-GFP was not affected under oxidative conditions. Recombinant GST-SV40 T-ag NLsc-GFP was prepared as described under “Experimental Procedures.” After a 2-h incubation of COS7 cells in the absence or presence of 2 mM H2O2, the recombinant GST-SV40 T-ag NLsc-GFP was microinjected into the cytoplasm, and then subcellular localization of GST-SV40 T-ag NLsc-GFP was examined at intervals of 1 min for 20 min (at least 20 cells were photographed per dish). Representative photographs are shown. Bar, 10 μm.
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Dependent mechanism even after ligand-dependent dissociation of hsp90.

Export of the GR from the Nucleus Is Not Affected under Oxidative Conditions—Because the trafficking of the GR between the cytoplasm and the nucleus is dynamic and bidirectional (31), the equilibrium of distribution of the GR is determined by the relationship between nuclear import and export rates. Compared with nuclear protein import, it is generally believed that the export kinetics is relatively slow. Therefore, nuclear protein transport appears to be strictly unidirectional in the short term (23). Predominantly cytoplasmic localization of the GR in the absence of hormonal DESG suggests that nuclear import is a rate-limiting step. Moreover, as shown in Fig. 6, export of GFP-hGR from the nucleus to the cytoplasm was not yet completed 24 h after withdrawal of dexamethasone and was not significantly affected by the addition of 0.5–2 mM H2O2. Thus, these results strongly suggest that treatment with H2O2 largely affects nuclear import of GFP-hGR.

Effect of Oxidative Conditions on Nuclear Import of SV40 T-ag-NLS—Next, we examined whether treatment with H2O2 affected the nuclear transport of the well characterized SV40 T-ag-NLS. We microinjected a recombinant chimeric protein, GST-NLS-GFP, which consists of GST, SV40 T-ag-NLS, and GFP, into the cytoplasm of COS7 cells (42). Regardless of the presence or absence of 2 mM H2O2, all cells so far examined revealed nuclear translocation of GST-NLSC-GFP within 15 min (Fig. 7), indicating that the cytoplasmic-nuclear transport machinery is not generally affected under oxidative conditions. Considering that NL1 but not the SV40 T-ag-NLS contains a cysteine residue that is well conserved among nuclear receptors (Fig. 8), we supposed that NLS of the GR, especially NL1, could be a target of redox-dependent modulation.

Reduction of Redox Sensitivity of the Human GR by Substitution of Cys-481 for Ser—To test the hypothesis that this conserved cysteine residue is a target of redox regulation, we substituted Cys for Ser at position 481 in the human GR to conserved cysteine residue is a target of redox-dependent modulation.

DISCUSSION

GFP has been developed as a protein tag for in situ and real time visualization of target proteins (75). We (26) and others (28, 33) have recently studied subcellular localization of the GR in living cells using GFP-hGR chimera. Here we again confirm that GFP-hGR exclusively docks in the cytoplasm in the absence of hormonal ligand and that nuclear translocation of GFP-hGR is strictly controlled in a ligand-dependent fashion. The kinetics of GFP-hGR translocation appears to be similar to those reported for native GR; the time for the half-maximum translocation (t1/2) is 5–10 min (Fig. 2, also compare with Refs. 24 and 27). Since not only ligand-dependent release of hsp90 and nuclear transport but also transcriptional function of GFP-hGR is maintained, this translocation assay system using GFP-hGR is strictly controlled in a ligand-dependent fashion. The kinetics of GFP-hGR translocation appears to be similar to those reported for native GR; the time for the half-maximum translocation (t1/2) is 5–10 min (Fig. 2, also compare with Refs. 24 and 27). Since not only ligand-dependent release of hsp90 and nuclear transport but also transcriptional function of GFP-hGR is maintained, this translocation assay system using GFP-hGR is strictly controlled in a ligand-dependent fashion.
cally controlled by redox-dependent mechanisms. Treatment of cells with H2O2 inhibits ligand-dependent nuclear translocation of the human GR, and treatment with NAC reverses this inhibitory effect of H2O2. We have also shown that oxidative treatment impairs ligand-dependent release of hsp90 from GFP-hGR. Given the putative importance of this chaperone protein for folding of the ligand binding domain (78), this effect may be closely associated with the reduction in ligand-binding activity of the GR in cultured cells under oxidative conditions (16, 17). Moreover, we showed that ligand-independent nuclear translocation of the GR is also inhibited under oxidative conditions. Thus, oxidative stress-mediated repression of GR nuclear import may not only be related to decreased ligand binding activity and subsequent impairment of release of hsp90, but may also be related to dysfunction of the nuclear translocation process itself. Under severe oxidative conditions, general dysfunction of subcellular organelles may occur, and the nuclear transport machinery itself may be generally damaged. Although GR NL1 is a bipartite motif and structurally distinct from canonical NLS motif such as that of SV40 T-ag (23), both NLS categories use, at least in part, similar transportation apparatus involving importins/karyopherins and Ran GTPase (23, 28, 79). Considering that nuclear localization of GFP-tagged SV40 T-ag NLS was not affected by treatment with H2O2, oxidative stress does not appear to generally repress the regulatory amino acids involved in redox-dependent intramolecular disulfide bond formation in the GR. For example, disulfide bond formation involving this cysteine residue may result in a protein conformation that hampers efficient interaction of the GR with NLS receptor proteins, e.g. importin-α/karyopherin-α even in the presence of ligands. Under reducing conditions, NL1 may interact with NLS receptor proteins in a ligand-dependent fashion, or alternatively, this may occur after liberation from hsp90, resulting in nuclear translocation of the GR. Although this cysteine residue is well conserved among nuclear receptors (Fig. 8), the functional significance has not yet been characterized for other receptor proteins. It has previously been shown that amino acid substitution of Cys-481 to Ser, contrary to amino acid substitution of Cys-481 to Arg (80), does not affect either DNA binding or transactivation functions of the GR (81). Therefore, it will be interesting to test whether this cysteine residue generally defines redox-dependent subcellular localization of nuclear receptors.

Although several reports have already documented that treatment with H2O2 of rat liver cytosol results in a decrease in ligand binding activity of the GR in vitro, we cannot compare the concentrations of H2O2 used in those studies (i.e. 20–100 mM in Refs. 12 and 82) with those used in the present in vivo study (i.e. 0.5–2 mM). We have also observed that sensitivity to H2O2 of nuclear translocation of GFP-hGR is extremely variable among distinct cell types (see also Ref. 83 for NF-κB activation), and steroid hormone-inducible gene expression is suppressed at lower concentrations of H2O2 in certain cells (16, 17, 84). Among the cells that we have studied, human mammary tumor cells ZR-75-1 were most sensitive to treatment with H2O2, and expression of reporter genes for either the estrogen receptor or the GR was affected under physiological concentrations of H2O2 (Ref. 84, and data not shown). Considering that reactive oxygen species including H2O2 are known to

FIG. 9. Substitution of Cys-481 for Ser reduced sensitivity of the human GR to oxidative treatment. A, GFP-hGR/C481S-expressing COS7 cells were incubated for 2 h in the absence (open circles) or presence of various concentrations of H2O2: 0.5 mM (filled triangles), 1 mM (open squares), and 2 mM H2O2 (filled circles). Then 100 nM dexamethasone was added. At the indicated time points after the addition of dexamethasone, subcellular localization of GFP-hGR/C481S was quantitatively assessed, and the percentages of the category N are shown. Experiments were repeated 3–5 times with almost identical results, and a representative graph is shown. B, COS7 cells were transfected with the expression plasmid for GFP-VP16-GR DBD/C481S fusion protein and cultured for 12 h in the absence or presence of H2O2. Then photographs were taken as described under “Experimental Procedures.”
be generated as a consequence of, for example, stimulation with cytokines (85) and phagocytosis (86, 87), repression of cellular glucocorticoid action under oxidative conditions may be physiologically important in inflammatory processes. To further confirm this model, redox sensitivity of the GR should be studied in a variety of cell types with reference to their physiological processes.

Together with our previous observations, we conclude that GR-mediated signals communicate with redox signals at multiple regulatory levels including ligand binding, nuclear translocation, DNA binding, and transcriptional activation. Although involvement of the cysteine residues is suggested in both cases, nuclear translocation of the transcription factor yAP-1, in clear contrast to the GR, is rather promoted in response to oxidative stress (88). Moreover, the multicyclop suppressor of SNF1 protein 2, Msnn2p, which contains two zinc finger motifs, translocates into the nucleus in response to a broad variety of stresses, e.g., exposure to heat shock, oxidative stress, ethanol, sorbate, and osmooress (89). Thus, redox-dependent modification of cysteine residues is considered to be one of the key regulatory mechanisms of protein localization within the cells. From a mechanistic point of view, it should be emphasized that not only oxidative stress but also hypoxic conditions influence subcellular compartmentalization of transcription factors. Notably, nuclear translocation of the hypoxia-inducible factor 1-α is promoted under hypoxic conditions (39). Therefore, cells may respond to alteration in oxygen tension via variable mechanisms including segregation of distinct transcription factors. In summary, the present results suggest that the dynamic cellular responses to redox state could play an important role of the glucocorticoid signal transduction mechanism.

Further analysis may be necessary to clarify not only underlying molecular mechanisms but also physiological significance as well.

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