Spatial Redox Regulation of a Critical Cysteine Residue of NF-κB in Vivo*

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Reduction-oxidation (redox) regulation has been implicated in the activation of the transcription factor NF-κB. However, the significance and mechanism of the redox regulation remain elusive, mainly due to the technical limitations caused by rapid proton transfer in redox reactions and by the presence of many redox molecules within cells. Here we establish versatile methods for measuring redox states of proteins and their individual cysteine residues in vitro and in vivo, involving thiol-modifying reagents and LC-MS analysis. Using these methods, we demonstrate that the redox state of NF-κB is spatially regulated by its subcellular localization. While the p65 subunit and most cysteine residues of the p50 subunit are reduced similarly in the cytoplasm and in the nucleus, Cys-62 of p50 is highly oxidized in the cytoplasm and strongly reduced in the nucleus. The reduced form of Cys-62 is essential for the DNA binding activity of NF-κB. Several lines of evidence suggest that the redox factor Ref-1 is involved in Cys-62 reduction in the nucleus. We propose that the Ref-1-dependent reduction of p50 in the nucleus is a necessary step for NF-κB activation. This study also provides the first example of a drug that inhibits the redox reaction between two specific proteins.

The redox states of cysteine residues, which can change reversibly within cells, often greatly influence the various properties of proteins, such as protein stability, chaperone activity, enzymatic activity, and protein structure (1-5). It has also been suggested that several transcription factors bind to their cognate sites in a redox-regulated manner. Well characterized examples include the prokaryotic transcription factors SoxR and OxyR, which function as oxidative stress sensors, their DNA binding activated through oxidation of critical cysteine residues (6-7). In most cases, however, the roles and mechanisms of redox regulation are not fully defined because it is difficult to monitor the alteration of redox states of proteins mainly due to the rapid proton transfer in redox reactions. A few have directly quantified the redox state of cysteine clustered with iron or amounts of oxidized cysteines using physicochemical or biochemical techniques (3, 8-9), but these methods cannot describe the whole picture of redox states of a protein and are not widely applicable to other proteins. Therefore, most researchers have chosen an indirect way of using cysteine-substitution mutant proteins (3-5, 7).

NF-κB is a eukaryotic transcription factor that regulates a wide variety of genes involved in immune function and development (10). NF-κB is composed of two subunits, p50 and p65, both of which are members of the Rel family of transcription factors. NF-κB normally exists in the cytoplasm, forming an inactive ternary complex with the inhibitor protein IκB. Following the application of appropriate stimuli, NF-κB is released from IκB and translocates into the nucleus, where it binds DNA and activates transcription of target genes. Mechanisms of NF-κB activation have been extensively studied; however, it is largely unknown if, and how, the DNA binding step is activated in cells. Some reports have described that the DNA binding activity of NF-κB is regulated by redox potential in vitro (11-13).

Recently, we reported an antiinflammatory drug that could allow us to solve this issue. The synthetic quinone derivative, (2E)-3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinonyl)-2-nonyl-2-propenoic acid (E3330), is a novel anti-NF-κB drug that specifically suppresses DNA binding activity of NF-κB but not those of other inflammatory transcription factors, such as activator protein-1 (AP-1) and nuclear factor of activated T cell (NF-AT), in phorbol 12-myristate 13-acetate (PMA)-stimulated Jurkat cells (14). Interestingly, E3330 did not affect the DNA binding activity of purified NF-κB or several steps of NF-κB activation, including IκB degradation, p65 phosphorylation, and nuclear translocation of NF-κB (14). This led us to hypothesize that E3330 may target an unknown nuclear factor that stimulates the DNA binding activity of NF-κB. Consistent with this assumption, we purified and identified redox factor-1 (Ref-1) from Jurkat nuclear extracts as an E3330-binding pro-

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tein that enhances DNA binding activity of NF-κB in an E3330-sensitive manner (15). Considering the specificity and affinity of the E3330-Ref1 interaction (15), E3330 is likely a selective inhibitor of Ref1 activity.

Ref1 is a nuclear protein that was originally identified as a DNA repair enzyme with an apurinic (AP)-endonuclease activity (16–18). Ref1 has been reported to stimulate activities of AP-1 as well as other transcription factors including p53 and hypoxia inducible factor-1α (18–24). In case of AP-1, it was well investigated by using in vitro cytotoxic point mutation analysis that DNA binding activity of AP-1 is stimulated by the reducing activity of Ref1. We have recently shown that Ref1 enhances the DNA binding activity of NF-κB in vitro as well as NF-κB-dependent transcriptional activation in vivo in an E3330-sensitive manner (15). However, few analyses have been performed to determine if these processes involve redox reaction, and how these processes occur in cells. The existence of many redox regulatory molecules, such as thioredoxin (Trx), thioredoxin-reductase (TrxR), glutaredoxin, nucleoredoxin, and glutathione, makes it difficult to explore the specific redox regulation between proteins in eukaryotic cells.

In order to overcome the difficulties associated with redox studies in vivo, we utilized E3330 as a useful tool because E3330 is a Ref1-specific inhibitor that specifically inhibits NF-κB activity but not the other transcription factors potentially regulated by Ref1 as mentioned above. Using irreversible thiol-modifying reagents and LC-MS analysis, we evaluated here the redox states of NF-κB during its activation step in vivo. We provide evidence that Cys-62 of NF-κB p65 is selectively reduced by Ref1 in the nucleus and that this reduction is a prerequisite for NF-κB activation in vivo.

EXPERIMENTAL PROCEDURES

Reagents—Thiol-modifying reagents, fluorescein-5-maleimide (F5M) and N-ethylnmaleimide (NEM), were obtained from Molecular Probes (Eugene, OR) and Nacalai Tesque (Kyoto, Japan), respectively, dissolved in dimethylformamide to a final concentration of 200 mM and stored at −20 °C until use. LysC was obtained from Wako (Osaka, Japan).

Recombinant Proteins—pET14b (Novagen)-based expression plasmids of full-length human p50 and Ref1 have previously been described (15). An expression vector of full-length human Trx was prepared by inserting the entire open reading frame amplified by polymerase chain reaction into the NdeI-BamHI sites of pET14b (Novagen). Each His-tagged protein was expressed in Escherichia coli strain BL21(DE3) and purified under native conditions using Ni-NTA-agarose beads (Qiagen) as instructed by the manufacturer. Purified proteins were dialyzed against dialysis buffer (HEPES pH 7.9, 100 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM F5M). After a 10-min incubation period, Nonidet P-40 was added to a final concentration of 0.6%, and tubes were mixed vigorously for 10 s. Tubes were centrifuged for 5 s at 10,000 rpm, and supernatants (cytoplasmic fractions) were removed by an additional separation on a packed cell volume of high salt buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM F5M. After a 20-min incubation period, tubes were centrifuged for 2 min at 15,000 rpm, and supernatants (nuclear fractions) were saved. F5M labeling reactions were stopped by adding 100 mM DTT to the cytoplasmic and nuclear fractions. Following immunoprecipitation using an anti-FLAG M2 affinity gel, eluates were subjected to SDS-PAGE, and fluorescence intensities of the proteins were analyzed as described above.

NEM Labeling and LC-MS Analysis—NEM labeling was performed similarly to F5M labeling, except that the experiments were performed with 1 mM (in vitro) and 20 mM (in vivo) NEM. Proteins were acetoneprecipitated, resuspended in a small volume of 8 M urea, and digested overnight at 37 °C with LysC in digestion buffer containing 25 mM Tris-HCl (pH 9.0), 2 mM urea, and 1% TFA. The high-performance liquid chromatography (HPLC)-coupled electron spray ionization time-of-flight MS system Mariner TK5000 (PerkinElmer) was used to analyze protein digests. Protein digests were initially separated on a protein/peptide ODS column with an inner diameter of 0.5 mm (PerkinElmer), using a linear gradient of 5–50% acetonitrile plus 1% formic acid at a flow rate of 60 μl/min. Peptide mass spectra were acquired online, using instrument settings for positive ion polarity at 3800 V spray tip potential, 130 V nozzle potential, 11.5 V skimmer 1 potential, 7.6 V quadrupole DC potential, 0.6 V deflection voltage, −37.5 V lens potential, 700 V quadrupole RF voltage, 140 °C quadrupole temperature, 140 °C nozzle temperature, 700 V push pulse potential, 275 V pull pulse potential, 5 V pull bias potential, 4000 V acceleration potential, 1400 V reflector potential, and 2150 V detector voltage. RNP is defined as (M_rNP/ (M_rN + M_rP)) × 100 (%), where M_rN and M_rP represent the mass intensities of NEM-labeled (M_rN) and -unlabeled (M_rP) peptides. M_rP partially comprises the mass intensity of hydrolytic-NEM-labeled peptide (<10% total NEM). For a single peptide, a semilog plot of RNP values was constructed with respect to the number of protons within the peptide (1–5H+). The RNP values obtained from peptides protonated differently were usually fairly similar. Figs. 2B and 4B show the averages of three or more RNP values.

Cell Culture—pcAGGS/FLAG-His-p50 (5 μg) and pCMVeos (0.5 μg) were co-transfected into Jurkat cells (5 × 10^6 cells) by electroporation. Cells were plated onto dishes containing RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum, 0.8% methylcellulose, and 300 μg/ml G418. After 3–4 weeks, G418-resistant clones were isolated, and expression of FLAG-His/p50 was examined by Western blotting using antibodies against p50 and FLAG. A FLAG-His/p50-expressing cell line of J-50 was maintained in RPMI 1640 containing 10% fetal calf serum and 200 μg/ml G418.

RESULTS

Assessment of the Thiol-trapping System by F5M—To measure the redox state of proteins, F5M, a maleimide derivative with a fluorescein group was employed as a thiol-modifying reagent. Maleimides irreversibly modify reduced, but not oxidized, cysteines rapidly at 4 °C at the physiological pH range (pH 6.5–8.0) where normal redox reactions can barely proceed. The thiol selectivity of maleimides is higher than those of other thiol-modifying reagents, such as iodoacetamides (25). Using F5M, the amount of proteins in the reduced state can be quantified as fluorescence intensity on an SDS-polyacrylamide gel (Fig. 1A). To assess this F5M-mediated thiol-trapping system, we initially examined a well characterized redox exchange
reaction between Trx and TrxR in vitro (26). In the presence of NADPH, TrxR specifically reduces Trx at the two conserved cysteine residues, Cys-32 and Cys-35. A constant amount of oxidized Trx was incubated with increasing concentrations of TrxR for various times at 37 °C, and reactions were processed for F5M labeling at 4 °C. As shown in Fig. 1, B and C, the fluorescence intensity of Trx increased with time, depending on the concentration of TrxR. When both Cys-32 and Cys-35 of Trx were mutated to serine residues, fluorescence intensity did not increase to a detectable level over time. These results strongly suggest that the F5M labeling reaction is specific and permits the quantitative measurement of the protein redox states.

Ref-1 Reduces p50, Leading to Activation of Its DNA Binding Activity in an E3330-sensitive Manner—We have previously shown using EMSA that Ref-1 stimulates DNA binding activity of p50/p50 homodimers and p50/p65 heterodimers, but not p65/p65 homodimers (15). These findings suggest that Ref-1 activates DNA binding of NF-κB by affecting the p50 subunit. To directly determine whether Ref-1 reduces p50, we used the above F5M labeling method. Oxidized and reduced forms of p50 were prepared by incubating recombinant p50 with the oxidizing reagent diamide and the reducing reagent TCEP, as described under “Experimental Procedures.” As shown in Fig. 2A, the reduced form of p50 (lane 1) but not the oxidized form (lane 2) strongly bound to the DNA probe containing an NF-κB binding site. When oxidized p50 was incubated with increasing amounts of recombinant Ref-1 (lanes 3–5), Trx (lanes 6–7), or TrxR (lanes 9–11) for 1 min prior to EMSA or F5M labeling, only Ref-1 reduced p50 and reactivated its DNA binding activity. Even in the presence of NADPH, TrxR was unable to reduce p50 under these conditions (lane 12). The level of reduction correlated well with the level of DNA binding.

To substantiate that redox exchange reactions occur between Ref-1 and p50, the redox states of both Ref-1 and p50 were monitored at different time points after mixing Ref-1 and oxidized p50. As shown in Fig. 1B, after 1 min, p50 was reduced to ~50% of the level achieved after a 60-min incubation. Conversely, fluorescence intensity of Ref-1 was decreased to ~60% in 1 min. We thus concluded that Ref-1 directly and rapidly reduces p50 by a redox exchange reaction.

It has been suggested, but not proven, that inhibition of Ref-1 by E3330 occurs through the block of its redox activity (15). Consistent with the previous results, E3330 suppressed Ref-1-dependent activation of DNA binding by oxidized p50 in a dose-dependent manner (Fig. 2C, upper panel, lanes 1–6). Similarly, E3330 also inhibited the Ref-1-dependent reduction of oxidized p50 (lower panel). In contrast, E3330 did not affect redox states or DNA binding activity of the reduced form of p50. From these results, we conclude that E3330 inhibition is mediated by the block of Ref-1 redox activity.

Significant Reduction of the Cys-62 Residue of p50 by Ref-1—The above method is a convenient way to measure changes in the redox status of proteins. However, it may not be suitable in cases where proteins contain multiple reduced cysteines of which only a subset is subject to redox regulation because F5M labeling of non-target cysteines may cause a high basal level of fluorescence.

Human p50 has seven cysteine residues at positions 62, 88, 119, 124, 162, 262, and 273. To identify cysteine residues of p50 that are reduced by Ref-1, we improved the above thiol-trapping method. The modified method comprises three steps: 1) irreversible labeling of reduced cysteines with another maleimide derivative, NEM, 2) digestion of NEM-labeled proteins by Lys-C endopeptidase, and 3) LC-MS analysis for the identi-
fication of NEM-labeled (reduced) and -unlabeled (oxidized) peptides. NEM was employed because F5M-labeled peptides could not be fully recovered from liquid chromatography, probably due to the hydrophobicity as a result of F5M labeling (data not shown). From the mass intensities of NEM-labeled and unlabeled peptides (MI\textsubscript{red} and MI\textsubscript{ox}), the fraction of reduced cysteine residues was estimated using the equation: MI\textsubscript{red}/(MI\textsubscript{red} + MI\textsubscript{ox}) \times 100 (%) or the ratio of NEM-labeled peptide (RNP).

From the results shown in Fig. 2A, we presumed that residues reduced by TCEP and Ref-1 should be functionally important. Recombinant p50 previously oxidized with diamide was incubated with TCEP or Ref-1 and processed as described above. Lys-C endopeptidase digests of p50 include three peptides containing a single cysteine residue and two with two cysteine residues (Table I). Fig. 3A shows representative mass spectra of the Cys-62-containing peptide. When oxidized p50 was left untreated, a large fraction of Cys-62 appeared to be fully oxidized because only the unlabeled peptide (53-77) was detected. Prior incubation with TCEP or Ref-1 resulted in the NEM-labeled peptide (53-77-NEM), indicating that Cys-62 was reduced by these reagents. The RPNs, obtained from their mass intensities, suggest that treatment of p50 with TCEP or Ref-1 resulted in 60 or 50% reduction of Cys-62 (Fig. 3B).

Similarly, all cysteine-containing peptides were identified by LC-MS analysis. RNP values for these peptides are presented in Fig. 3B. For peptides containing two cysteines, RPNs were calculated for singly and doubly labeled peptides, respectively. All the cysteine residues were reduced by Ref-1 by varying degrees; however, the reduction of Cys-62 by Ref-1 was significant and highly sensitive to E3330 inhibition. For comparison, EMSAs for an identical set of reactions were performed (Fig. 3C). The DNA binding activity of p50 correlated well with the redox states of Cys-62, indicating that Cys-62 plays a determinant role in DNA binding. Unexpectedly, TCEP did not reduce all the cysteine residues of p50. TCEP may be capable of reducing only the exposed cysteine residues under the non-denaturing condition. Consistent with this hypothesis, structural data show that thiol groups of Cys-88 and Cys-162, which were not reduced by TCEP in our experiments, are buried in the p50 protein (27).

The Reduced Form of p50 Cys-62 Is Essential for Its DNA Binding Activity—The above results do not exclude the possibility that, in addition to Cys-62, other cysteines may play some role in redox regulation of p50. To address this issue, we carried out EMSA and fluorescence assays for point mutants of p50, whose cysteine residues at positions 62, 119, 262, and 273 were

| Positions | Sequence | Number of NEM labeling | Theoretical mass (Z1) |
|-----------|----------|------------------------|----------------------|
| 53–77     | QRGRFRFYVCEGPSPHGLPGAS SEK | 0 | 2722.33 |
|           |          | | 2847.45 |
| 87–95     | ICNYVGPAAK | 0 | 964.49 |
|           | | | 1089.62 |
| 118–131   | HCEDGICTVTAGPK | 0 | 1430.64 |
|           | | | 1555.77 |
| 162       | VFTLLEMTACIRGYPGLLV HPDLAYLQAEGGDRQGDREK | 0 | 1680.90 |
|           | | | 1890.90 |
| 253–275   | IVRMDRTAGCVTGGEEIVA LCDK | 0 | 2542.85 |
|           | | | 2667.37 |
|           | | | 2792.50 |
individually changed to serines (C62S, C119S, C262S, and C273S). Serine was chosen because it may mimic a reduced form of cysteine. Similar sets of the point mutants were previously described and analyzed by EMSA (12, 28). As observed for wild type p50, DNA binding activities of C119S, C262S, and C273S were not detectable under oxidized conditions and were strongly stimulated by TCEP in a concentration-dependent manner (Fig. 4). In sharp contrast, C62S showed strong DNA binding even in its oxidized form and was not affected by TCEP, i.e. by the redox states of the other cysteine residues. The results of EMSA are consistent with those of earlier reports (12, 28, 29). Taken together, it is very likely that Cys-62 alone is responsible for Ref-1-mediated reduction and activation of p50.

Reduction of p50 by Ref-1 in the Nucleus—Using these redox-monitoring methods, we investigated the possibility that the redox state of NF-κB is regulated during the activation process in vivo. We established a Jurkat cell line expressing p50 fused to FLAG and 6× His tags at the N terminus (J-50 cells) to obtain a sufficient quantity of homogenous NF-κB complex from cells. FLAG-His-p50 (FLAG-p50) behaved similarly to endogenous p50, i.e. it formed a ternary complex with p65 and IκBα in the cytoplasm, was translocated into the nucleus on PMA treatment concomitant with IκBα degradation, and bound DNA as a heterodimer with p65 in a sequence-specific manner (data not shown).

To visualize the overall redox state of NF-κB in cells, we modified the F5M labeling protocol as follows: J-50 cells were maintained with or without PMA for 30 min, labeled in situ with membrane-permeable F5M, lysed, and fractionated into cytoplasmic and nuclear fractions. From these fractions, tagged NF-κB complexes were immunoprecipitated with anti-FLAG antibody and separated on SDS gels. Protein amounts and fluorescence intensities of FLAG-p50 and p65 were measured by silver staining and fluorescent imaging of the gels (Fig. 5). Relative fluorescence intensity was calculated by normalizing the fluorescence intensity to its protein amount, and values of cytoplasmic FLAG-p50 and p65 in unstimulated cells were set as 100%. Because a very small amount of FLAG-p50 was present in the nucleus of unstimulated cells, the corresponding sample was concentrated 6-fold before analysis. F5M labeled the FLAG-p50 protein more strongly in the nucleus than in the cytoplasm of untreated cells (Fig. 5A, lanes 3 and 9). In contrast, the relative fluorescence intensity of p65 was similar in both the cytoplasm and nucleus. PMA stimulation of cells increased nuclear p50 and p65 protein amounts, but did not significantly affect their overall redox states, regardless of subcellular location (Fig. 5, A and B, lanes 3, 4, 9, and 10). These results indicate that p50 is reduced upon translocation into the nucleus and that this reduction occurs independently of extracellular stimuli. To our knowledge, this is the first report demonstrating different redox states of a protein in the nucleus and cytoplasm.

![Image](https://via.placeholder.com/150.png?text=Image1)
What is the protein(s) responsible for the observed reduction of p50 in the nucleus? Among the various redox regulatory proteins, Trx and Ref-1 have been implicated in the modulation of NF-κB activity (12, 13, 30). We therefore employed E3330 to investigate the possible role of Ref-1 in the reduction of p50 in the nucleus. As shown in Fig. 5A, treatment of cells with increasing concentrations of E3330 prior to PMA stimulation inhibited p50 reduction in the nucleus, but not in the cytoplasm. In contrast, E3330 had little effect on the redox states of p65 (Fig. 5B). These results indicate that Ref-1 is involved in p50 reduction in the nucleus but not in the reduction of cytoplasmic p50/p65 or nuclear p65.

Redox Regulation of Cys-62 in Vivo—We next examined the redox states of individual cysteine residues of p50 using a combination of in vivo NEM labeling and LC-MS analysis. Following sequential treatment with E3330 and PMA, J-50 cells were labeled in situ with NEM and separated into cytoplasmic and nuclear fractions. These extracts were sequentially subjected to anti-FLAG affinity chromatography and nickel chelate chromatography under denaturing conditions to yield homogenous FLAG-p50 (Fig. 6B, top left panel). Very small amounts of FLAG-p50 were obtained from the nuclear fraction without PMA treatment, and therefore no further analyses were performed on this sample. Purified FLAG-p50 was digested with Lys-C endopeptidase and subjected to LC-MS, and the data were processed similarly to Fig. 3. Fig. 6A shows the representative mass spectra of the Cys-62-containing peptide. In the cytoplasm of untreated or PMA-treated cells, a large fraction of p50 Cys-62 was oxidized, as observed from the mass intensity of unlabeled peptide (53-77), which was higher than that of the NEM-labeled peptide (53-77-NEM). In sharp contrast, p50 in the PMA-treated cell nucleus contained significantly more reduced Cys-62, evident from the relative mass intensity of NEM-labeled and unlabeled peptides. All the cysteine-containing peptides were similarly identified by LC-MS analyses, and RNP values for these peptides are presented in Fig. 6B. These results indicate that half or more of the Cys-88, Cys-119, Cys-124, and Cys-162 residues are reduced constitutively, regardless of the subcellular location of NF-κB or the presence of PMA or E3330. In sharp contrast, the level of Cys-62 reduction appears to be low (~19%) in the cytoplasm and high (~80%) in the nucleus. Similarly, Cys-262/273 reduction is low-to-moderate (3–57%) in the cytoplasm and moderate-to-high (30–80%) in the nucleus. These results indicate that nucleus-specific reduced state of p50 observed in Fig. 4A is due to the reduction of these three cysteine residues. Strikingly, the increased reduction of Cys-62 was severely impaired by prior treatment of the cells with E3330, resulting in a reduction level far below that found in the cytoplasm (Fig. 6, A and B). In contrast, the reduction of Cys-262/273 in the nucleus was not substantially affected by E3330. It is therefore likely that Ref-1 plays a major role in the reduction of Cys-62 in

Fig. 4. The reduced form of Cys-62 is critical for DNA binding by p50. Oxidized forms of p50 wild type (WT) and its mutants were incubated with increasing concentrations of TCEP for 1 h at 37 °C. Their DNA binding activities and redox states were analyzed by EMSA and fluorescence assays. C62S, C119S, C262S, and C273S represent p50 mutants carrying cysteine to serine substitutions at positions 62, 119, 262, and 273.

Fig. 5. Visualization of the redox states of NF-κB in vivo. A and B, J-50 (2 × 10⁷) cells were incubated with the indicated concentrations of E3330 for 2 h, followed by 50 μg/ml PMA for 30 min where specified. Cells were labeled with F5M and fractionated as described under “Experimental Procedures.” FLAG-NF-κB complexes were immunoprecipitated from the cytoplasmic and nuclear fractions using an anti-FLAG M2 affinity gel, eluted with FLAG peptide (500 μM), resolved by SDS-PAGE, and visualized by fluorescence scanning or silver staining. The relative fluorescence intensities normalized to protein amounts are shown at the bottom. Immunoprecipitation from control Jurkat cells resulted in undetectable fluorescence (lanes 1 and 2).
the nucleus but not of the other cysteine residues of p50. These findings, together with the observation that E3330 inhibits NF-κB-dependent transcriptional activation (14), strongly suggest that Ref-1-mediated reduction of p50 Cys-62 in the nucleus is a prerequisite for NF-κB activation.

**DISCUSSION**

**Ref-1 Activates NF-κB through Reduction of p50**—We showed here that Ref-1 specifically reduced Cys-62 of p50, which led to activation of NF-κB DNA binding in vitro and in vivo. We also showed that Trx and TrxR had no effect on the redox status of p50. Previous studies have implicated Trx in the redox regulation of NF-κB (12–13, 30). However, several lines of evidence suggest that Ref-1 plays a major role in p50 reduction in vivo. In vitro, a 2.5-fold molar excess of Ref-1, but not of Trx, reduced and activated p50 in 1 min (Fig. 2A), suggesting that Ref-1 possesses a higher reducing potential than Trx. Previous studies used a 1000-fold molar excess of Trx and a 15- or 30-min incubation to see activation of NF-κB (12, 30). In addition, Trx predominantly localizes in the cytoplasm (31). Quantitative Western blotting suggests that Ref-1 exists at 0.5–1 × 10⁶ molecules per cell or 100- to 200-fold molar excess to p50, whereas Trx exists at 0.1–0.2 × 10⁶ molecules per cell or 20- to 40-fold molar excess in the nucleus of PMA-stimulated Jurkat or J-50 cells.² It was previously reported that cytoplasmic Trx translocates into the nucleus upon stimulation of cells with PMA or UV light (31–32); however, we did not observe any detectable change in Trx localization even 2-h post-PMA stimulation (data not shown). Because p50 in the nucleus became reduced within 30-min post-PMA stimulation and because this reduction was strongly inhibited by E3330 (Figs. 5A and 6B), it seems unlikely that Trx directly reduces p50 in the nucleus, at least under our experimental conditions. Trx may contribute to NF-κB activation indirectly by enhancing Ref-1 redox activity (31, 33) or by regulating IκB degradation in the cytoplasm (34).

Our data are consistent with a recent report showing that induction of Ref-1 expression coincides with the activation of DNA binding by NF-κB, whereas inhibition of Ref-1 expression by an antisense oligonucleotide abolishes DNA binding activity of NF-κB in granule neurons (35).

Ref-1 targets p50 but not its heterodimeric partner p65. p50 (436 amino acids) and p65 (552 amino acids) show significant sequence similarity over the Rel homology region (RHR) spanning −300 amino acids at their N termini (~40% identity and ~50% similarity). The RHR, which encodes two functions, DNA

² T. Nishi and H. Handa, unpublished observations.
binding and dimerization, is important for formation of the stable NF-κB-DNA complex. Why does Ref-1 selectively reduce p50? We have previously shown using GST pull-down assays that Ref-1 physically interacts with p50 but not with p65 (15). Because the sequence around p50 Cys-62, the primary target of Ref-1, is highly conserved among other members of the Rel family, it is unlikely that the short sequence determines substrate specificity. More detailed structural analysis is necessary to elucidate the specific interaction between Ref-1 and p50.

**Spacio-temporal Regulation of p50 Reduction**—We also found that the redox states of p50 are regulated by its subcellular location (Fig. 5A). Of the seven cysteine residues, Cys-62 and Cys-262/273 are primary targets of nucleus-specific reduction (Fig. 6). Given their differential sensitivity to E3330, it is likely that Cys-62 and Cys-262/273 are reduced primarily by Ref-1 and another redox molecule(s), respectively, in the nucleus. Why are these residues oxidized more strongly in the cytoplasm? Paradoxically, Ref-1, which is responsible for Cys-62 reduction in the nucleus, is present both in the cytoplasm and in the nucleus (data not shown). Possibly, Ref-1 redox activity may differ with its location, and cytoplasmic Ref-1 may be incapable of reducing p50 Cys-62. This is consistent with our results of *in vivo* fluorescence assays showing that Ref-1 is in a more oxidized state in the cytoplasm (unpublished data). Similarly, redox molecules responsible for Cys-262/273 reduction may exist as active forms only in the nucleus. Alternatively, reduction of these residues may be inhibited in the cytoplasm by the presence of another factor, possibly IκB. In this regard, it should be noted that Cys-273 lies within the IκB-binding domain (36–37). IκB binding to NF-κB may directly block the access of redox molecules to the target cysteines or may induce its conformational change (36) such that the redox molecules cannot access the target cysteines. The two possibilities above are not mutually exclusive, and both may contribute to the prevention of Cys-62 and Cys-262/273 reduction in the cytoplasm.

**The Unique Characteristics of p50 Cys-62**—Using LC-MS, we demonstrated that activation of DNA binding by NF-κB is caused by the reduction of p50 Cys-62. What is the structural basis for the redox regulation of Cys-62? According to the crystal structure of NF-κB in a complex with DNA, Cys-62 is located in the N-terminal DNA recognition loop (27, 38, 39). Because the sequence around p50 Cys-62, the primary target of Ref-1, is highly conserved among other members of the Rel family, it is unlikely that the short sequence determines substrate specificity. More detailed structural analysis is necessary to elucidate the specific interaction between Ref-1 and p50.

What type of oxidation occurs at Cys-62 *in vivo*? We could not see it because all the naturally oxidized forms of cysteine are reduced at the DTT treatment step in the current protocols. This reduction step, which is employed (i) to terminate NEM labeling to prevent unwanted NEM modification of other amino acids, and (ii) to increase the efficiency of the subsequent LysC digestion, was critical for obtaining reasonable MS data of p50 (data not shown). However, the use of reducing agents may be avoided by using other reagents and procedures. For example, NEM labeling reactions may be terminated by gel filtration, and the efficiency of proteolytic digestion may be increased by using other alkylation agents and proteases. Possible types of cysteine modification include inter- or intramolecular disulfide bond formation, sulfenic acid intermediate formation (S-OH), and S-nitrosylation (S-NO). Among them, S-nitrosylation is a newly discovered, reversible modification involving nitric oxide (NO) and may be relevant to oxidation of p50 Cys-62 (40–43).

**Effective Methods for Measuring Protein Redox States**—In this study, we have established effective methods for measuring the redox state of a given protein using maleimides. We believe that the results obtained by these methods reflect the actual redox states of p50 because one of the main conclusions of this study is that Cys-62 is the primary redox target within cells. This agrees with a number of reports showing its importance by indirect ways using point mutants *in vitro* (12, 25–26). One might argue that the redox states of p50 could change during the isolation of the protein from the cells. However, this is not likely because we did not observe any detectable change in the redox states of recombinant p50 that was either oxidized or reduced during processing using the same techniques as were used for Jurkat cell extracts (data not shown).

It should be noted that there is some ambiguity in the results obtained by these methods. First, NEM labeling may affect the quality of the LC-MS data by changing the physicochemical property of peptides, possibly by changing the column yield and the ionization efficiency. Second, the RNP values can underestimate the reduction levels of corresponding cysteines if the efficiency of F5M or NEM labeling is not 100%. Labeling efficiency may be greatly affected by location of the protein within the cell or by location of the cysteine within the protein. Thus, it cannot be concluded that the RNP values represent exact ratios of reduced cysteines. For the former point, more accurate comparison will be made by including several internal standards. Concerning the latter point, we think that this is not a serious problem, at least for p50, because Cys-124, which is buried in the p50 protein (27), was labeled by NEM at an efficiency of 40–80% *in vivo* (Fig. 6B). Since F5M and NEM are small hydrophobic molecules, they may invade various cellular compartments or even the inside regions of proteins, thereby labeling the reduced cysteines at high efficiency. In sum, the current methods allow us to obtain semi-quantitative, interpretable data and to draw a reasonable model that can be tested by other approaches.

In theory, the methods described here are applicable to virtually any cellular protein. Some drawbacks include the possible ambiguity of the results as discussed above and the difficulty of obtaining a sufficient quantity (>100 pmol) of pure proteins from cells for LC-MS analysis. In this study, a 16-liter culture of J-50 cells was used for each analysis. The protein amounts needed for these experiments can be greatly de-
creased by improving the sensitivity of LC-MS analysis. That said, these methods are the versatile ways of monitoring the redox states of cysteines in vitro and in vivo and provide an additional opportunity for the study of redox regulation.

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