Thioltransferase (Glutaredoxin) Reactivates the DNA-binding Activity of Oxidation-inactivated Nuclear Factor I*

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The reversible oxidative inactivation of transcription factors has been proposed to be important in cellular responses to oxidant stress and in several signal transduction pathways. The nuclear factor I (NFI) family of transcription factors is sensitive to oxidative inactivation due to the presence of a conserved, oxidation-sensitive cysteine residue within the NFI DNA-binding domain. Here we show that restoration of the DNA-binding activity of oxidized NFI-C can be catalyzed in vitro by the cellular enzyme thioltransferase (glutaredoxin) coupled to GSH and GSSG reductase. To test whether GSH-dependent pathways play a role in the maintenance of NFI activity in vivo, we used buthionine sulfoximine, an agent that inhibits GSH synthesis, and N-acetylcysteine, an agent that can replenish intracellular GSH. Pretreatment of HeLa cells with buthionine sulfoximine greatly potentiated the inactivation of NFI by the oxidizing agent diamide. Inclusion of N-acetylcysteine in the culture medium during the recovery period following diamide treatment increased the extent of restoration of NFI activity. These results suggest that maintenance of the DNA-binding activity of NFI proteins during oxidant stress in vivo requires a GSH-dependent pathway, likely involving thioltransferase-catalyzed reduction of the oxidation-sensitive cysteine residue on NFI.

Modulation of DNA-binding activity by oxidation and reduction reactions (redox regulation) has been demonstrated for a number of transcription factors, including NF-kB/Rel proteins, Fos and Jun proteins, and others (1–6). The mechanism of oxidative inactivation of DNA binding is through the oxidation of specific cysteine residues close to or within the DNA-binding domains of these proteins. This modulation of the DNA-binding activity of transcription factors by redox state has been proposed to be important both in cellular response to oxidant stress and in a number of signal transduction pathways including those mediated by tumor necrosis factor α (7, 8). While the process of oxidative inactivation of DNA binding has been studied extensively in vitro, the biochemical pathways that mediate the reversible oxidation of oxidation-sensitive cysteine residues are only poorly understood. We are using the NFI family of transcription factors as a model system to characterize the pathways for reversible oxidative inactivation of DNA-binding proteins in vitro and in vivo.

The NFI family of site-specific DNA-binding proteins is required both for adenovirus DNA replication and for the expression of a large number of cellular and viral genes (9–11). In vertebrates, this family of proteins is encoded by four distinct genes (NFI-A, NFI-B, NFI-C, and NFI-X), and the expression patterns of the four genes are regulated during cell differentiation and development (12, 13). We had shown previously that the DNA-binding activity of the NFI family of transcription factors was inactivated by oxidation in vitro (14). This sensitivity to oxidation requires the presence of a single oxidation-sensitive cysteine residue that is conserved within the DNA-binding domains of all NFI proteins from Caenorhabditis elegans to humans. Mutation of this oxidation-sensitive cysteine residue creates NFI proteins with apparently normal activity; however, modification of these cysteines, e.g. by glutathionylation, alters the activity of the protease in a reversible fashion (15, 16).

If the modulation of DNA-binding activity by redox state is a regulated physiological process, we would anticipate that specific cellular proteins would mediate the oxidation and reduction of oxidation-sensitive cysteine residues on DNA-binding proteins. Indeed, there are two classes of proteins in mammalian cells that have been shown to mediate the reduction of oxidation-sensitive cysteine residues on DNA-binding proteins in vitro. Studies from several laboratories have demonstrated that the bifunctional HAP1/Ref-1 protein can reactivate oxidized Fos/Jun proteins in vitro (17, 18). This redox activity is present in the N-terminal domain of HAP1/Ref-1 and is separate from the C-terminal domain of HAP1/Ref-1 that mediates repair of DNA damage (19). Also, the cellular reducing protein thioredoxin has been shown to reactivate oxidized NF-xB in vitro (2, 3). Thioredoxin was first discovered as the protein that provided reducing equivalents to ribonucleotide reductase and has been shown to mediate the reduction of a number of intracellular proteins (20). Likewise, thioltransferase (glutaredoxin) has also been shown to catalyze the reactivation of many oxidatively modified proteins. In particular, we found that thioredoxin (TTase) is specific for protein-glutathione mixed

ferase; GMSA, gel mobility shift assay; DTT, dithiothreitol; BSO, bu-thionine sulfoximine.
disulfides (protein-SSG) (21, 22), and the catalytic intermediate TTase-SSG is selectively recycled to the reduced enzyme by GSH (23), with formation of GSSG and regeneration of GSH by coupling with NADPH and GSSG reductase. These characteristic interactions of thiolttransferase with glutathione distinguish it from thioredoxin, which favors intramolecular disulfide substrates and is turned over by NADPH and thioredoxin reductase, independently of glutathione.

Since oxidation of the single cysteine residue on NFI in the presence of GSH likely forms NFI-SSG in vitro or in vivo, we tested the ability of thiolttransferase to restore the DNA-binding activity of NFI that was oxidized by diamide in the presence of GSH. Here we report that thiolttransferase can reactivate oxidized NFI protein in vitro in a GSH-dependent manner. In addition, we demonstrate that treatment with buthionine sulfoximine, a known inhibitor of GSH synthesis (24), potentiates the oxidative inactivation of NFI in cultured HeLa cells and that inclusion of N-acetylcysteine (precursor of intracellular GSH) in the culture medium during the recovery period following diamide treatment increases the extent of restoration of the DNA-binding activity of NFI. These findings suggest an important role for thiolttransferase and intracellular GSH in the reduction of oxidation-sensitive cysteine residues in the NFI family of transcription factors and potentially other site-specific DNA-binding proteins.

MATERIALS AND METHODS

Recombinant NFI Expression and Purification—Construction of the pET220 vectors that express the NH2-terminal 220 amino acids of wild-type or mutant human NFI-C from a T7 promoter was described previously (25). All of the NFI proteins expressed contained a hexahistidine tag fused to the N terminus of the human NFI-C protein. Expression of wild-type and mutant NFI proteins in Escherichia coli cells and preparation of bacterial extracts were as described (14). The NFI proteins were purified by absorption to phosphocellulose equilibrated with 25 mM Hepes, 10% sucrose, 350 mM NaCl, 0.1% Nonidet P-40, and 2 mM DTT and eluted with the same buffer containing 1.0–1.5 M NaCl. The final purification step was achieved by Ni2+–nitrilotriacetic acid-agarose chromatography as described previously (14). Protein concentration was determined by the method of Bradford (26). Protein preparations were analyzed by electrophoresis on 10% SDS-polyacrylamide gels, determined to be >90% pure by silver staining (Bio-Rad), and quantified using bovine serum albumin as a standard.

Gel Mobility Shift Assay (GMSA)—NFI DNA-binding activity was determined by GMSAs with purified recombinant proteins or extracts of nuclei using the 32P-labeled FIB-2.6 oligonucleotide or a control mutant oligonucleotide (FIB-2.6C2) as described (25). The FIB-2.6 oligonucleotide contains a well characterized NFI-binding site cloned previously from human genomic DNA, whereas the FIB-2.6C2 oligonucleotide has a single point mutation that reduces NFI binding >100-fold in vitro. The inactivation of NFI DNA-binding activity by the oxidizing agent diamide and the reactivation by reducing agents or the TTase-reducing system were performed as indicated in the figure legends. NFI-DNA complexes were quantified using a Molecular Dynamics Model 400 PhosphorImager.

Preparations of Thiolttransferase—Thiolttransferase was purified from human red blood cells as described (27), and recombinant human thiolttransferase was purified from E. coli (28); both sources of the enzyme display the same catalytic characteristics (28) and are used interchangeably. One unit of thiolttransferase is defined as the amount of enzyme required to catalyze the glutathione-dependent reduction of 1 mol of cysteinyglutathione disulfide/min under standard assay conditions in which GSSG formation is coupled to NADPH oxidation by GSSG reductase (21, 28). The reactivation of the DNA-binding activity of oxidized NFI was demonstrated with purified human thiolttransferase isolated in both ways.

SDS-Polyacrylamide Gel Electrophoresis and Immunodetection—SDS-polyacrylamide gel electrophoresis was performed on 10–12.5% gels as described previously (29). For immunodetection, proteins were transferred to Immobilon-P membrane (Millipore Corp.) and probed with an affinity-purified polyclonal rabbit antiserum raised against the amino-terminal NFI-C220 peptide acetyl-DEHPFPFIEALLC. The NFI proteins were detected by chemiluminescence as described (24).

Cell Culture and Preparation of Nuclear Extracts—HeLa cells were cultured in α-minimum essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in 5% CO2. To induce oxidative stress, cells were treated with 10 mM diamide at 37 °C for 15–30 min and washed with phosphate-buffered saline as described in the figure legends. Subsequent recovery of the cells was performed for 2 h at 37 °C in the presence or absence of the protein synthesis inhibitor cycloheximide (25 μg/ml). To deplete the cells of glutathione, cells were incubated with 100 μM buthionine sulfoximine (BSO) for 48 h. BSO blocks the synthesis of glutathione by inhibiting the enzyme γ-glutamylcysteine synthetase (24). More than 90% of the cells were viable under this condition. Cells were treated with diamide as described above and allowed to recovery from oxidant stress in the presence and absence of 25 mM N-acetylcysteine. For the preparation of nuclear extracts, the cells were scraped into cold phosphate-buffered saline and harvested. The cells were lysed, and nuclear extracts were prepared in 25 mM Hepes, pH 7.5, 10% sucrose, 0.01% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride containing 0.35 M NaCl as described previously (30).

RESULTS

Our earlier study showed that Cys-3 present in the DNA-binding domain of all NFI proteins was sensitive to oxidation and chemical modification in vitro (14). Oxidation inactivates the DNA-binding activity of NFI, and subsequent treatment with the nonphysiological reducing agent DTT restores the binding activity. Since GSH is a major intracellular reductant and is believed to play a role in the maintenance of reduced sulphydryl groups on proteins in vivo (31), we tested whether reduced GSH alone could restore the DNA-binding activity of the oxidized NFI-C220 protein in vitro in comparison with the nonphysiological reducing agents DTT and β-mercaptoethanol. As shown previously, purified NFI-C220 binds specifically to the FIB-2.6 oligonucleotide, which contains a consensuss NFI-binding site (Fig. 1, lane 2). Treatment of NFI-C220 in vitro with 1 mM diamide abolished the DNA-binding activity (Fig. 1, lane 3). As expected, this inactivation was reversed when the oxidized NFI protein was further incubated with a 5-fold molar excess of the nonphysiological reducing agent DTT (Fig. 1, lane 4) or β-mercaptoethanol (lane 5). However, a 5-fold molar excess of GSH failed to reactivate oxidized NFI under these conditions (Fig. 1, lane 6), and GSSG also had no effect, as expected (lane 7). The lack of an effect of GSH alone in reactivating oxidized NFI-C220 is similar to observations with other sulphydryl proteins whose activities are inhibited by oxidation and not restored by GSH alone. For example, oxidatively inactivated phosphofructokinase and glutathione S-transferase are not reactivated by GSH unless thiolttransferase is also present (22), indicating that rapid reactivation requires enzyme catalysis.

Thiolttransferases have long been implicated in the maintenance of sensitive intracellular thiols and the reduction of protein disulfides (22). To determine whether TTase can regenerate the DNA-binding activity of oxidized NFI, NFI protein was incubated with diamide, diluted in the presence of GSH to quench the diamide, and then incubated in the absence or presence of thiolttransferase. As shown previously, the DNA-binding activity was abolished when NFI was oxidized with diamide (Fig. 2, lane 3 versus lane 1). With subsequent incubation in the presence of a 4-fold molar excess of GSH alone or GSH plus NADPH and GSSG reductase, a low level of binding activity was restored (Fig. 2, lanes 4 and 7, respectively). This limited reactivation by GSH at 30 °C was not observed when the incubation was performed at 4 °C (Fig. 1, lane 6). A much greater restoration of DNA-binding activity was seen when oxidized NFI was incubated with thiolttransferase in the presence of GSH and the GSH-regenerating system (Fig. 2, lane 6). This increased reactivation required both TTase and the regenerating system since incubation with either component alone produced levels of DNA-binding activity similar to that seen.
with GSH alone (Fig. 2, lanes 7 and 8). The extent of NFI reactivation by TTase was only slightly less than that obtained with the nonphysiological reducing agent DTT (Fig. 2, lane 5). In the absence of oxidation, GSH had no effect on the binding activity of NFI (Fig. 2, lane 2), and as expected TTase, did not affect the DNA-binding activity of either the non-oxidized wild type or the oxidation-resistant Cys-3 mutants of NFI (data not shown). These data indicate that TTase, in the presence of GSH and a GSH-regenerating system, can effectively reactivate the DNA-binding activity of diamide-oxidized NFI. On a molar basis, thioltransferase (0.7 μM) is at least 3000-fold more efficient than GSH alone (2 mM) and 1200-fold better than DTT (1 mM) in effecting NFI reactivation under the same conditions.

Previously, we had shown that the DNA-binding activity of NFI is inactivated by oxidation in vitro (14). To determine whether the DNA-binding activity of NFI is sensitive to oxidative inactivation in intact cells, we measured NFI DNA-binding activity in extracts of HeLa cells treated with diamide (Fig. 4). HeLa cells (<50% confluent) were cultured in the presence of 10 mM diamide and harvested, and nuclear extracts were prepared as described under “Materials and Methods.” As shown previously (30), nuclear extracts from untreated HeLa cells yielded multiple specific NFI-DNA complexes (Fig. 4, lane 4). NFI DNA-binding activity was reduced by >75% after diamide treatment of HeLa cells for 30 min (Fig. 4, lane 5). When the diamide-treated HeLa cells were washed and cultured for a further 2-h recovery period in the absence of diamide, there was an ~2-fold increase in NFI DNA-binding activity (Fig. 4, lane 6), but the level of binding activity remained below that seen in control cells. This partial restoration of NFI DNA-binding activity appears to be independent of protein synthesis since incubation of HeLa cells with the protein synthesis in-
 Activation of Nuclear Factor I by thioltransferase

The oxidation of recombinant NFI-C220 by diamide and subsequent incubation in the presence and absence of TTase were performed as described under “Results.” To measure the time course of reactivation, diamide-oxidized NFI was incubated at 30 °C for 5, 10, 15, or 30 min with identical amounts of appropriate reagents including TTase (0.016 units) and GSH (0.2 mM) or the GSH-regenerating system as described in the legend to Fig. 2. The samples were then incubated with 1 nmol of [35S]labeled FIB-2.6 oligonucleotide for 20 min at 4 °C and analyzed by electrophoresis. NFI DNA complexes were quantified on a PhosphorImager and are expressed in arbitrary relative density units. The amount of DNA-binding activity is plotted versus the time of incubation. The inset shows the dependence of NFI DNA-binding activity reactivation on TTase concentration in the presence of GSH and the GSH-regenerating system for a 5-min reactivation time. The percent of NFI DNA-binding activity recovered is relative to DTT-reactivated NFI (100%).

Since intracellular glutathione is a major redox buffer in cells and a known substrate for thioltransferase (21–23) and may protect proteins from oxidative inactivation (31, 32), we investigated the potential role of glutathione in the oxidative inactivation and subsequent recovery of NFI DNA-binding activity. HeLa cells were cultured with two agents known to inactivate and subsequent recovery of NFI DNA-binding activity after oxidant stress.

The inefficiency of GSH alone in reactivating oxidized NFI recovery of activity appear to be cell density-dependent and that little or no reactivation of binding occurs unless >70% inactivation of NFI is achieved during the initial diamide treatment (data not shown). This apparent cell density dependence may be due to previously observed differences in GSH levels in low versus high density cells in actively growing cultures (33, 34). These results indicate that intracellular GSH levels may affect both the sensitivity of NFI to oxidative inactivation and the subsequent recovery of NFI DNA-binding activity after oxidative stress.

**DISCUSSION**

While our previous study showed the existence of a highly conserved, oxidation-sensitive cysteine residue in the NFI family of transcription factors (14), the physiological significance of the residue remained unclear. Here we demonstrate both that the in vitro oxidative inactivation of NFI-C can be reversed by treatment with the cellular enzyme thioltransferase (Figs. 2 and 3) and that NFI DNA-binding activity is subject to oxidative inactivation and reactivation in intact cells (Figs. 4 and 5). The inefficiency of GSH alone in reactivating oxidized NFI (Figs. 2 and 3), together with the potentiation of the oxidative
inactivation of NFI in intact cells by treatment with an agent shown to deplete intracellular GSH (BSO) (Fig. 5), suggests that GSH may be necessary for the protection and/or restoration of NFI DNA-binding activity in vivo, but it is probably not sufficient. Hence, the ability of TTase to catalyze GSH-dependent reactivation of NFI DNA-binding activity in vitro suggests that TTase may play an important role in the regulation of NFI DNA-binding activity. Hence, the ability of TTase to catalyze GSH-dependent reduction of protein-SSG substrates (21, 22), its catalytic effectiveness in reducing oxidized NFI in the presence of GSH is consistent with NFI-SSG being the actual substrate for TTase. This interpretation is based on extensive studies of TTase catalysis of GSH-dependent reduction of model protein mixed disulfides, including cysteinyI, cysteaminyl, and glutathionyl mixed disulfides of hemoglobin, papain, and bovine serum albumin (21, 22). In addition to the generation of mixed and intramolecular disulfides, the formation of stable sulfenic, sulfenic, or sulfonic acids by monothiol oxidation has also been proposed as a mechanism for the oxidative inactivation of a number of proteins (1, 17, 38). In this context, in vitro treatments of glyceraldehyde-3-phosphate dehydrogenase with H2O2 and tetrathionate and of phosphofructokinase with GSSG were designed to give glyceraldehyde-3-phosphate dehydrogenase-S-OH, glyceraldehyde-3-phosphate dehydrogenase-S-SO3-, and phosphofructokinase-SSG as the corresponding inactivated forms of the enzymes (39). Comparison of equimolar amounts of TTase and thioredoxin near their physiological concentrations indicated that TTase was much more effective than thioredoxin for reactivating phosphofructokinase-SSG, as would be predicted from the substrate selectivity of TTase that we delineated (21, 22). Thioredoxin, however, was better for reactivating the two types (sulfenate and thiosulfate) of modified glyceraldehyde-3-phosphate dehydrogenase (39).

By analogy to this comparison, it appears less likely that monothiol oxidation plays a role in the oxidative inactivation of NFI. In other studies in which monothiol oxidation has been proposed as a means of inactivation, the redox proteins thioredoxin (2, 3) and HAP1/Ref1 (16–18) were reported to effectively reactivate the oxidized proteins. In preliminary experiments, we did not observe reactivation of diamide-oxidized NFI with typical concentrations of the reduced form of either HAP1 or thioredoxin; however, broader studies are necessary to define the relative effectiveness of TTase and these other redox proteins (data not shown; proteins generously provided by Drs. I. Hickson and A. Gronenborn, respectively). The apparent differences in the protein requirements for the reductive reactivation of different intracellular proteins may reflect distinct biochemical pathways used to prevent or recover from oxidative stress. For example, E. coli encodes at least three different proteins capable of donating reducing equivalents to ribonucleotide reductase, each of which may also function in the reduction of oxidized intracellular proteins (40). It will be necessary in the future to selectively deplete each of the known potential redox regulators in eukaryotic cells to assess their different functions and their potential for synergism in vivo.

Our data demonstrate that NFI DNA-binding activity is sensitive in vivo to oxidative inactivation particularly under conditions of glutathione depletion (Fig. 5). However, it remains to be determined how glutathione participates in this process. Besides reacting directly with diamide or other oxidative mediators, GSH is likely to be incorporated into NFI molecules during oxidative stress to form NFI-SSG, and then the GSH would be removed by dethiolation by TTase. In fact, we have observed incorporation of [35S]GSH into NFI in the presence of diamide (data not shown). This observation is consistent with the fact that oxidant treatment of a broad variety of cells (hepatocytes, neutrophils, gastric mucosal cells, etc.) generally has produced 35S-glutathionylated proteins (41–47), and...
it has been suggested that S-thiolation during oxidative stress might protect particular proteins from irreversible damage (46, 47).

The oxidative inactivation of the DNA-binding activity of NFI in intact cells and the effects of the GSH modulators BSO and N-acetylcysteine (Figs. 4 and 5) suggest that inactivation of NFI can occur during oxidative stress in vivo, and the extent and duration of the changes in NFI activity would be dependent on the intracellular GSH status. For example, oxidative stress occurs in various physiological and pathological states, including human immunodeficiency virus infection, where it is believed to be the result of chronic exposure of the infected cells to inflammatory cytokines (48). The oxidative situation associated with the infection may foster inflammation since oxidative stress is believed to be an important factor in disease (49, 50). Future experiments involving expression of transfected cDNA for TThase may provide more direct evidence for a role of this enzyme in regulating oxidatively damaged proteins in situ, including NFI and human immunodeficiency virus type 1 protease. In this regard, thioltransferase has been shown to display site selectivity in deglutathionylation of Cys-95 and Cys-67 of the protease (16).

Whether analogous conditions of oxidative stress can lead to the inactivation of NFI DNA-binding activity in situ is under investigation. In addition, it would be of interest to see whether the oxidative inactivation of NFI family members occurs early in apoptosis since oxidative stress is believed to be an important event during apoptotic cell death (49, 50). Future experiments involving expression of transfected cDNA for TThase may provide more direct evidence for a role of this enzyme in regulating oxidatively damaged proteins in situ, including NFI and human immunodeficiency virus type 1 protease.

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