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Forum Review

Oxidation of Zinc Finger Transcription Factors: Physiological Consequences

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ABSTRACT

Redox-sensitive cysteine residues are present in the interaction domains of many protein complexes. There are examples in all of the major categories of transcription factors, including basic region, leucine zipper, helix–loop–helix, and zinc finger. Zinc finger structures require at least two zinc-coordinated cysteine sulfhydryl groups, and oxidation or alkylation of these can eliminate DNA-binding and transcriptional functions. We review here the evidence for oxidation of zinc finger cysteines, the pathways and reactive oxygen intermediates involved, and the functional and physiological consequences of these reactions. Despite skepticism that the strongly reducing intracellular environment would permit significant oxidation of cysteine residues within zinc finger transcription factors, there is compelling evidence that oxidation occurs both *in vitro* and *in vivo*. Early reports demonstrating reversible oxidation of zinc-coordinated cysteines with loss of binding function *in vitro* were shown to reflect accurately the changes in intact cells, and these in turn have been shown to correlate with physiological changes. In particular, the accumulation of oxidized Sp1 zinc fingers during aging, and estrogen receptors in tamoxifen-resistant breast cancers are dramatic examples of what may be a general sensitivity of zinc finger factors to changes in the redox state of the cell. Antioxid. Redox Signal. 3, 535–548.

INTRODUCTION

ZINC FINGER PROTEINS appeared early during the evolution of eukaryotes, possibly coevolving with pathways that regulate intracellular zinc homeostasis (13, 19). Genome analyses show that the proportion of zinc finger coding sequences increases with genome complexity and may be as high as 1% of mammalian genomes (55). There are several classes of zinc fingers determined by the amino acids that coordinate the zinc. The most common types of zinc finger contain two cysteines and two histidines or four cysteines in the zinc coordination domain, and >1,000 such specific zinc finger sequences have been reported. Included in this group are transcription factor IIIA (TFIIIA), multiple nuclear hormone receptors, and the ubiquitous Sp1 family of factors. All of the common zinc finger proteins contain two or more zinc-coordinated cysteines, and in general the cysteine must be in the reduced form. Oxidation of the cysteine thiol group within a zinc finger eliminates zinc coordination, disassembles the secondary structure, and destroys the DNA binding properties. This is most likely to happen under conditions of elevated oxidative stress when the redox potential of the cell cytoplasm is increased. The consequence is reduced transcription of affected genes. Examples of cysteine thiol group oxidation have been reported in all classes of tran-

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scription factors indicating that metal-coordinated groups are not essential for oxidation (52, 80). Oxygen-derived free radicals may simultaneously modulate electrophilic attack on cysteine residues and stimulate protein kinase cell signaling pathways (44, 81). The activated signaling pathways usually mediate increased gene transcription, and there is emerging evidence that zinc fingers are involved at several levels in this phenomenon, possibly as redox sensors (2, 9, 26, 47, 71, 87). The superoxide radical is probably the principal diffusing species of reactive oxygen (5, 29, 62), and all intracellular redox reactions are buffered by soluble antioxidant and enzyme pathways that coevolved to maintain a reducing intracellular environment (75). The purpose of this review is to summarize the evidence for redox regulation of zinc finger transcription factors, describe the features that determine the reactivity of zinc finger cysteines in these proteins, and present evidence that this regulation is physiologically relevant.

REVERSIBLE OXIDATION OF TRANSCRIPTION FACTOR CYSTEINE RESIDUES

The initial evidence for redox regulation through transcription factor cysteines came from *in vitro* studies using cell extracts and purified proteins. Critical cysteine residues contained in the DNA binding sites of the glucocorticoid receptor (GR), activator protein 1 (Ap1), nuclear factor-κB (NF-κB), and c-myb were shown to be susceptible to attack by oxidizing and alkylating agents (1, 27, 28, 35, 36, 84, 85, 91, 92). The relevant cysteine residues are contained in the zinc fingers of GR and the leucine zipper and basic DNA-binding regions of AP1 and c-myb. Abate *et al.* (1) showed that binding of AP1 (c-Jun) was modulated by the redox state of a single conserved cysteine residue. Similar observations were made on the redox sensitivity of GR, NF-κB, and c-myb binding (28, 84, 85). In all of these studies, electrophoretic gel mobility shifts or footprints were used to examine the effects of oxidizing agents, the alkylating agent *N*-ethylmaleimide, and reducing agents on the binding of nuclear extracts or proteins synthesized *in vitro*. In all cases, oxidative inactivation involved the formation of intraprotein disulfides. Abate *et al.* (1) also identified a nuclear protein, subsequently named redox factor-1 (Ref-1), that promoted reduction of the essential c-Jun cysteine residue in the presence of reduced thioredoxin. The target for NF-κB oxidation was suggested to be the cytosolic DNA-binding subunit, present in an inactive form in noninduced cells. The authors of these reports concluded that redox regulation of critical transcription factor cysteines may be an important form of transcriptional control for some genes.

The physiological relevance of protein thiol oxidation *in vitro* was questioned by Staal *et al.* (78, 79), Meyer *et al.*, (64) and Schreck *et al.* (73), who considered that the presence of antioxidants in the cell cytosol, in particular millimolar concentrations of glutathione, may be incompatible with the direct redox regulation of these factors in intact cells. Staal *et al.* (78, 79) reported that, rather than losing DNA binding through oxidation, NF-κB was strongly induced by oxidative stress *in vivo*. In response to this, Toledano and Leonard (85) conceded "although both (our studies and those of Staal *et al.*) are consistent with a potential role for oxidation–reduction in the control of NF-κB, they at least superficially disagree in their conclusions." Meyer *et al.* analyzed the effects of anti- and prooxidants on AP1 and NF-κB binding activity and gene regulation *in vitro* and *in vivo*. They concluded that these factors predominantly mediated increased transcription and protein synthesis in response to changes in the redox state of the cell.

The human HoxB5 and *Escherichia* coli OxyR are examples of transcription factors that are activated by cysteine oxidation (10, 24, 95). The HoxB5 protein binds to DNA *in vitro* when either oxidized or reduced, but binds cooperatively only when oxidized. Mutational analysis revealed that an essential cysteine residue in the turn between homeodomain helices was necessary for cooperative binding and redox regulation. The OxyR transcription factor activates *E. coli* antioxidant defense genes in response to elevated hydrogen peroxide (H_2O_2). It is inactive in the reduced form; activation requires thiol oxidation and formation of an in-

tramolecular disulfide bond between conserved cysteine residues. Reduction of the disulfide by reduced glutathione (GSH), a reaction catalyzed by glutaredoxin, breaks this bond and inactivates OxyR. The OxyR system is a classical example of a redox sensor where the participating cysteine residues are in equilibrium with cytosolic GSH/oxidized glutathione (GSSG), and become oxidized and activated when the cytosol redox potential is increased. It also illustrates an elegant physiological role for reversible oxidation of cysteine thiols in activating antioxidant defenses.

REDOX-RESPONSIVE ZINC FINGER FACTORS

Zinc finger proteins are the largest classes of DNA-binding proteins (12, 38, 69). The identification of zinc finger cysteines as redox-sensitive structures was first reported for GR, early growth response factor-1 (Egr-1), and Sp1 (34, 35, 51). GR, like other steroid hormone receptors, contains two zinc fingers in which the zinc is coordinated with four cysteines (Cys_4); Sp1 and Egr-1 each contain three zinc fingers of the Cys_2His_2 class and recognize a common GC-rich DNA-binding site (50). DNA binding of all three proteins was found to be sensitive to oxidizing agents and alkylating agents *in vitro*. Egr-1 synthesized *in vitro* was shown to have an absolute requirement for dithiothreitol (DTT) or β-mercaptoethanol to bind DNA, suggesting that it was synthesized in an oxidized inactive form. Nuclear extracts from HeLa cells or purified Ref-1 also activated binding of *de novo* synthesized Egr-1 by catalyzing the reduction of cysteine disulfides. The second zinc finger of Egr-1 has the sequence Gln-Cys-Arg, a consensus for interaction with Ref-1 (51). Knoepfel *et al*. (48) described the redox sensitivity of Sp1 and confirmed that DNA binding strongly protected Sp1 from both oxidizing and alkylating agents. They found that removal of zinc from Sp1 rendered the protein highly susceptible to oxidation within a physiological range of cellular redox change. These results suggest that *de novo* synthesized Egr-1 and Sp1 proteins may be particularly sensitive to oxidation, whereas the DNA-bound proteins are less sensitive. Clearly the levels of intracellular zinc and its availability may play roles in determining the oxidation state of zinc fingers. There is strong evidence that zinc levels are under the control of metallothioneins (MTs) that sequester and release intracellular zinc in a redox-dependent manner (39, 41, 59). MTs are a large family of cysteine-rich proteins involved in heavy metal detoxification and antioxidant defense, as well as zinc homeostasis (39, 42, 58). They contain seven sites that reversibly bind zinc; interestingly, MT gene transcription is induced by metals, redox stress, and hypoxia indicating a complex pattern of autoregulation (8, 65).

The physiological relevance of Cys_2His_2 zinc finger oxidation was questioned again by Nose and Ohba (66), who found that contrary to the effects of oxidizing agents *in vitro*, osteoblastic MC3T3 cells responded to oxidative stress by activating Egr-1 expression, as well as Egr-1-dependent transcription. They showed that the treatment of cells with up to 200 μM H_2O_2 increased Egr-1 protein content and binding activity of nuclear extracts and induced a five-fold increase of Egr-1-dependent transcription of a transfected promoter. Again it was suggested that the highly reduced intracellular environment prohibits the direct redox regulation of zinc finger transcription factors such as Egr-1.

The strong induction of Egr-1 expression (as well as NF-κB and AP1) in response to oxidative stress is the consequence of stress-activated protein kinase cascades (for reviews, see 53, 62, 72, 75, 81). The conflicting results on the effects of oxidative stress can be explained if transcriptional activation under some treatment conditions masks the effect of direct oxidation on factors such as Egr-1 and AP1. Alternatively, different degrees of oxidative stress may be required for the oxidation of zinc finger cysteines and activation of kinase cascades. As one approach to determine whether zinc finger proteins are direct targets for oxidation *in vivo*, our group analyzed Sp1 binding in redox-stressed cells and nuclear extracts and correlated this binding with transcription from both transfected and endogenous Sp1-dependent promoters (90). Sp1 was chosen because it is essential for "housekeeping" gene expression

and is not induced transcriptionally by oxidative stress. We confirmed previous reports that the binding *in vitro* of Sp1, Egr-1, and NF-κB, but not serum response factor (SRF), was sensitive to oxidation by diamide and this was reversible by treatment with DTT. We also demonstrated that DNA-bound Sp1 was partially protected from oxidation, and the redox sensitivity was related to the apparent binding affinity of Sp1 to DNA. Sp1 binds to high- and low-affinity G/C-rich binding sites, and binding to the low-affinity site conferred less protection as may be predicted from a weaker interaction (90). Taking these binding studies a step further, we demonstrated that a strong oxidative stress, involving chronic exposure of cells to L-buthionine-[*S*,*R*]-sulfoximine (BSO) followed by acute diamide treatment, conditions that depleted soluble thiols by >80%, caused the selective oxidation of Sp1 and Egr-1. Nuclear extracts from these cells lost all detectable low-affinity Sp1 binding; there was a >50% loss of Egr-1 binding, but no detectable change of SRF or AP1 binding.

To determine whether it was possible to demonstrate selective repression of zinc finger-dependent transcription by oxidative stress *in vivo*, we subjected HeLa cells and C_2C_{12} skeletal myocytes to a strong acute oxidative stress, and measured the transcription rates of Sp1-dependent transfected and endogenous genes. Exposure of HeLa cells to BSO (24 h) and diamide (4 h) resulted in a selective loss of transcription from the Sp1-dependent simian virus 40 promoter with no loss of transcription from control (non–Sp1-dependent) promoters. The endogenous housekeeping genes β-enolase and dihydrofolate reductase (DHFR) have critical Sp1 sites in the proximal promoters that functionally replace TATA-box elements to direct transcriptional initiation. Mutation of these Sp1 elements almost eliminates transcription. We found that the endogenous transcripts of both β-enolase and DHFR genes were dramatically reduced under strong oxidative stress conditions, whereas heme oxygenase and MT transcripts increased. Similar patterns were observed with transfected promoters; β-enolase and DHFR promoters were repressed by oxidative stress, whereas heme oxygenase and MT promoters were induced. To confirm that Sp1 was the target we also demonstrated that a β-enolase promoter with mutations of the Sp1-binding site was no longer sensitive to oxidative stress (90). These experiments provided strong evidence that zinc fingers were susceptible physically and functionally to oxidative stress in intact cells.

Other groups reported that the GR and estrogen receptors (ER) were also sensitive to oxidation in intact cells (21, 30, 54). Esposito *et al*. (21) transfected COS cells with an expression vector coding for the GR and treated the transfected cells with diethyl maleate or BSO to deplete intracellular GSH. Nuclear extracts isolated from these cells lost GR-DNA binding activity, and the same conditions inhibited the expression of a transfected GR-regulated promoter. Similarly, Hayashi *et al*. (30) found that the expression of both endogenous and transfected genes normally regulated by the ER in ZR-75-1 breast cancer cells was highly sensitive to H_2O_2 treatment in the range 20–200 μM (the same range that activated Egr-1, see above). Hayashi *et al*. demonstrated that both ER DNA binding activity and ER-dependent gene expression could be protected by simultaneous overexpression of the thioredoxin gene. The latter observation supported earlier work that demonstrated interactions between GR and thioredoxin in rat liver cytosol extracts (27, 84).

Together these results provided strong evidence that zinc finger transcription factors, in particular Sp1 and steroid receptors, can be oxidized and inactivated selectively by hyperoxidizing conditions *in vitro* and *in vivo*. But there still remains the question of physiological relevance; whether the results obtained by treating proteins or cells with oxidizing agents represent changes that occur within the physiological range of oxidative stress. As discussed earlier, these reactions, although clearly possible under experimental conditions, may be effectively quenched by antioxidants in tissues under normal physiological states.

EQUILIBRIUM OF THIOLS WITH OXIDATIVE AND ANTIOXIDATIVE PATHWAYS

The probability that a zinc-coordinated cysteine will be subjected to electrophilic attack by

reactive oxygen is determined by the physical location of the protein, redox potential of the thiol group, accessibility of the cysteine within the protein, and the redox potentials of the proximal antioxidants. We have already cited an example in *E. coli* OxyR, where the redox potential of a regulatory cysteine residue is close to the redox potential of the cytosol, so that physiologically relevant changes of the latter determine the oxidation state of the former. Figure 1 summarizes the major oxidation and antioxidant pathways of mammalian cells that determine the cytosolic redox potential. There are three major sources of superoxide production (Fig. 1, I–III at left); the relative magnitudes of each are determined by the physiological context. The activity of pathway I is usually increased in tissues subjected to ischemia and reperfusion (for review, see 88). Hypoxanthine accumulates in ischemic tissue due to the catabolism of ATP; reperfusion favors the conversion of xanthine dehydrogenase to xanthine oxidase and stimulates the reaction shown in the figure. The mitochondrial electron transport chain (Fig. 1, pathway II) is the major source of reactive oxygen in most cells and is particularly active in cells with high-energy turnover, such as cardiac muscle. The mitochondrial electron transport chain can leak up to 2% of electrons directly to molecular oxygen generating superoxide, mainly from the NADH-DH-complex I and the ubisemiquinone-cytochrome b_1c complex II (4, 5, 61, 76, 96). In the final pathway (III), NADPH oxidase is activated, mostly in neutrophils, eosinophils, and polymorphonuclear phagocytes, by the so-called oxidative burst. In resting cells, the components of NADPH-oxidase, including $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, reside in the cell cytosol (for review, see 32). On activation, $p47^{phox}$ and $p67^{phox}$ associate with the cell membrane, where they can interact with cytochrome b558 and catalyze the transfer of electrons from NADPH to molecular oxygen.

Mitochondrial and cytosolic superoxide dismutases (SODs) convert superoxide to H_2O_2 and water. H_2O_2 is subsequently converted to water by the actions of catalase or other peroxidases, or it may react with transition metals (Fe^{2+}) to produce hydroxyl free radicals, $OH^{\cdot}$. Superoxide can react with locally produced nitric oxide to generate peroxynitrite $OOONO^-$ (89). H_2O_2 and superoxide are the major diffusible species (4, 5, 61, 76, 96); $OH^{\cdot}$ and $OONO^-$ are more reactive with shorter half-lives. Reactive oxygen species that escape mitochondrial and cytosolic SOD/catalase/peroxidase systems can oxidize lipids, proteins, and DNA. The different intracellular targets of superoxide and hydroxyl radicals are illustrated by the distinct kinase pathways that are activated in cells treated with superoxide or H_2O_2 (97). The rate of production of reactive oxygen and the corresponding oxidation of intracellular components contribute to the overall redox potential of the cytosol. In particular, the ratio GSH:GSSG is decreased by oxidative stress. Cellular glutathione is present in millimolar concentrations and is in equilibrium with accessible protein cysteine thiols. As indicated in Fig. 1, because of its high intracellular concentration, glutathione probably mediates many intracellular redox interactions involving oxygen free radicals and cellular proteins. Increased levels of GSSG promote thiol–disulfide exchange reactions and oxidize cellular proteins. Some zinc finger sites, for example, GR, ER, and Egr-1, may be selectively reduced by interaction with members of the family of oxidoreductase enzymes that include thioredoxin, glutaredoxin, nucleoredoxin (33, 56, 57), and Ref-1 (92).

ZINC FINGER TARGETS FOR SUPEROXIDE AND THIOL–DISULFIDE EXCHANGE

Studies on the OxyR and SoxR transcription factors establish a role for cysteine residues as physiological redox sensors in *E. coli* (10, 95). A similar physiological role for cysteine has been determined from analyses of the differential redox sensitivities of zinc-coordinated cysteine residues in mammalian MTs (39, 40, 41, 59). It is also clear from the discussion so far that some zinc fingers can be selectively oxidized *in vitro* and *in vivo* in experimental models, and may be responsive to physiological changes of GSH and GSSG (16, 39). The latter possibility is determined by the molecular structure of the zinc finger and its physical location.

The most common zinc finger transcription

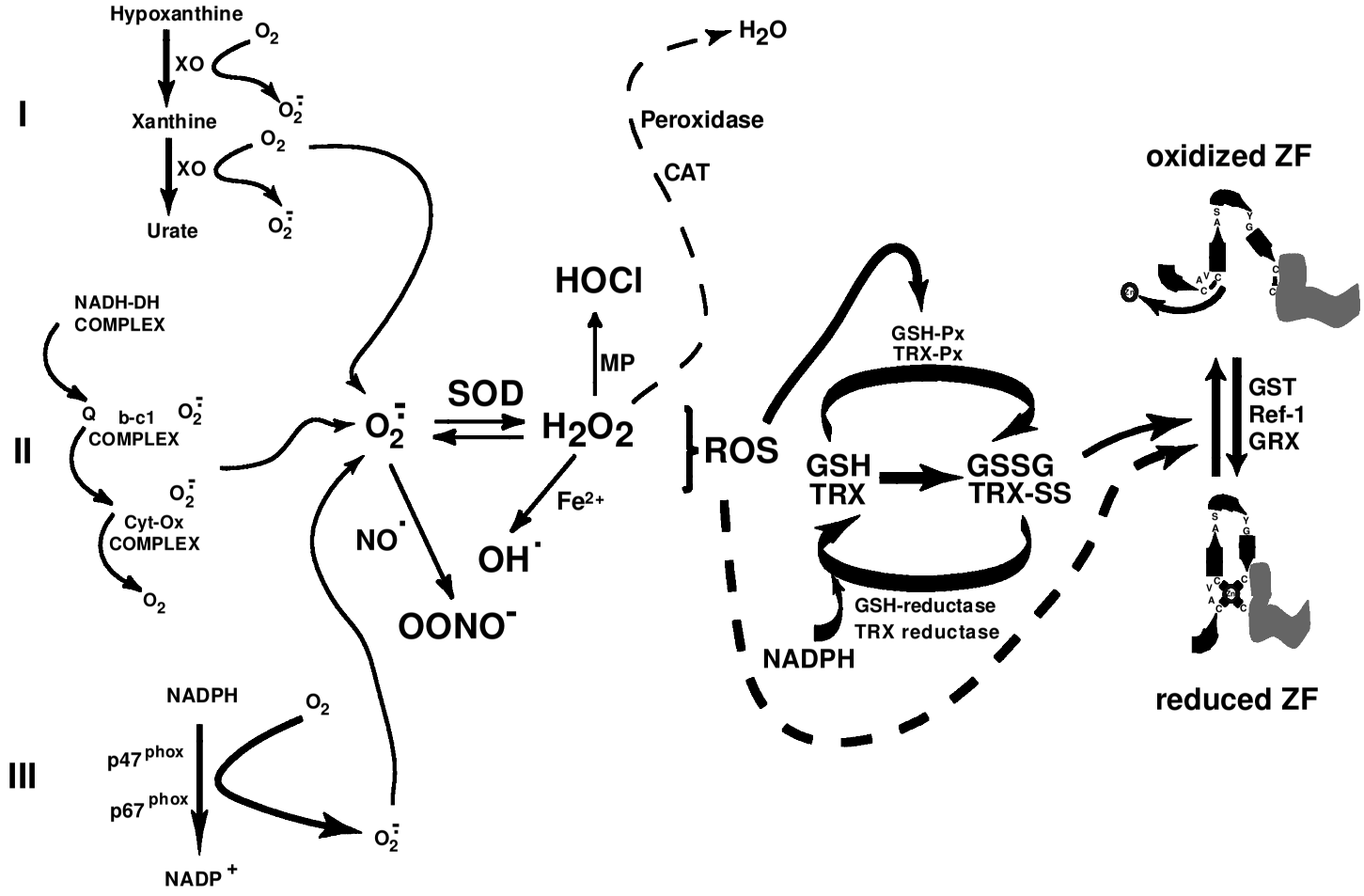

FIG. 1. Major oxidant and antioxidant pathways in mammalian cells. Pathways I–III on the left show the three main sources of reactive oxygen. Superoxide ($O_2^{\cdot}$) is generated by xanthine oxidase (XO) during conversion of hypoxanthine to urate in the final stages of nucleotide catabolism (89). The mitochondrial electron transport chain (II) is probably the main source of reactive oxygen species (ROS) in most cells under most conditions. The final pathway (III) is especially evident in neutrophils, eosinophils, and polymorphonuclear phagocytes. Mitochondrial and cytosolic superoxide dismutases (SODs) convert superoxide to H_2O_2 that is neutralized by catalase (CAT) and glutathione peroxidases (GSH-Px), [or may be converted to hypochlorous acid (HOCl) by myeloperoxidase (MP) in neutrophils (32)]. Mitochondrial Mn-SOD and cytosolic Cu,Zn-SOD are the main forms of SOD. Elevated levels of superoxide in the cytosol result in oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) and the formation of intramolecular disulfide bonds. This reaction may be mediated by GSH-Px and thioredoxin peroxidase (TRX-Px). Increased concentrations of GSSG stimulate thiol–disulfide exchange with cellular proteins, resulting in thiol conversions to disulfides, and disruption of protein structure in the case of zinc fingers. The dashed arrow represents direct oxidation of zinc fingers by ROS, considered to be a less frequent event. GSSG is converted back to GSH by GSH-reductase and/or thioredoxin (TRX)-reductase in reactions coupled to NADPH. At far right is a schematic representation of a reduced and oxidized Cys_4 zinc finger (ZF) (see Fig. 2, below). There is some evidence that the oxidation of disulfides may be reversed by the oxido-reductase enzymes glutaredoxin (GRX), TRX, Ref-1, and glutathione *S*-transferase (GST). NADH-DH, nicotinamide adenine dinucleotide dehydrogenase; $NO^{\cdot}$, nitric oxide; $OH^{\cdot}$, hydroxyl radical; $OONO^{-}$, peroxynitrite.

factors are the Cys_2His_2 (TFIIIA, Sp1, Egr-1) (20, 43) and Cys_4 (GATA factors, ER, and GR) classes, although at least nine different classes within the Cys/His family are recognized (12, 22, 86). Consensus structures of the major zinc finger classes are illustrated in Fig. 2. In the Cys_2His_2 class, the pairs of cysteines and histidines are separated by a loop of 12 amino acids of the form (Tyr,Phe)-X-Cys-X_{2-4}-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His, where X represents more variable amino acids. The structure of each zinc finger domain consists of two antiparallel β strands followed by an α-helix. Seven or more fingers may contribute to DNA binding specificity. The overall Cys_4 structure is quite similar with cysteine residues also separated by β strands and α-helices.

The reactivity of a cysteine sulfhydryl (with GSH/GSSG) within a zinc finger is determined primarily by the neighboring two or three amino acids, in combination with the overall redox potential of the surrounding medium, and the concentration of glutathione. Aromatic neighbors in the primary amino acid sequence stabilize disulfides by forming S-π complexes, and favor oxidation. Neighboring basic amino acids such as histidine also favor disulfide exchange (77). All charged amino acids influence the pK values electrostatically and can strongly influence the frequency of collision between intracellular cysteines and disulfides, again determining their reactivity. Snyder *et al*. (77) determined that the net charge at the protein cysteine site is the sum of the single charges of the thiolate and the charges of the amino acids immediately preceding and following the cysteine in the primary sequence. They estimated that at pH 7.0 and physiological ionic strengths, rate constants for reaction of a disulfide with a cysteine having two positive neighbors, one positive and one neutral neighbor, or two neutral neighbors are 132,000, 3,350, and 367 $s^{-1}\ M^{-1}$. Therefore, it can be estimated that cysteine local environments may dictate a 10^6-fold range in rate constants of disulfide reactions within and between proteins.

For a significant fraction of zinc finger sulfhydryl groups to become oxidized in equilibrium with the thiol/disulfide status of the cytosol, the oxidation potential of the zinc finger cysteine(s) must be close to the thiol/disulfide status of the cytosol as determined by the GSH:GSSG ratio. The susceptibility of a cysteine to thiol–disulfide exchange has been determined in GSH:GSSG redox buffers and defined as the ratio of GSH:GSSG required to keep a protein half-reduced at equilibrium (16). This value has been determined for a number of proteins and varies over 12 orders of magnitude; for example, GSH:GSSG ratios of 2×10^{-5} and 10^6 have been reported to maintain a half-reduced state of glycogen phosphorylase and chloroplast fructose-1,6-biphosphatase, respectively. Many proteins, including Sp1, fall well within the physiological range of cellular GSH:GSSG change (63). In the liver, GSH:GSSG ratios as high as 400 have been reported under nonstressed conditions and <10 under conditions of glutathione depletion or drug-induced oxidative stress (16, 77). GSH:GSSG ratios close to 1 have been reported in the endoplasmic

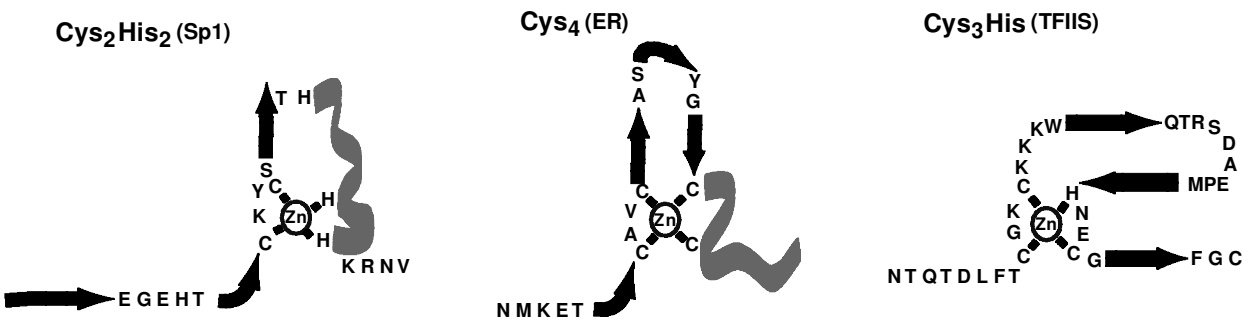

FIG. 2. Topologies of the major zinc finger transcription factors Cys_2His_2, Cys_3His, and Cys_4. Black bars with arrowheads represent β-sheet structures; gray areas are α-helices. Structures and sequences are based on Sp1, ER, and TFIIS (74).

reticulum (37). The three Sp1 zinc fingers have the following sequences: **Gln His** Ile Cys **His** Ile **Gln** Gly Cys Gly **Lys** Val (ZF1); **Pro Phe** Met Cys Thr **Trp** Ser **Tyr** Cys Gly **Lys Arg** (ZF2); **Lys Phe** Ala Cys **Pro** Glu Cys **Pro Lys Arg** (ZF3). Therefore, fingers 1–3 have three, two, and three basic amino acids respectively, flanking the cysteines, and fingers 2 and 3 have four and three aromatic amino acids respectively. The ER zinc fingers have the sequence Thr **Arg Tyr** Cys Ala Val Cys **Asn** Asp Tyr (1) and Val **Trp** Ser Cys Glu Gly Cys **Lys** Ala **Phe** (2), with one basic amino acid and two aromatic amino acids for each finger (see Fig. 2). Therefore, the microenvironment of each of these zinc fingers is compatible with enhanced reactivity.

Studies on human MT (hMT) provide a precedent for thiol–disulfide exchange reactions at zinc-coordinated cysteine sulfhydryls within a physiological range of change in GSH:GSSG (39, 59). In hMT, 20 cysteine residues bind seven zinc atoms in two clusters such that each zinc atom has four cysteine ligands. The zinc–cysteinyl interactions are of two types, including bridging and terminal thiolates, and the α and β clusters differ in the number and types of these bonds. The molecular architecture confers extremely high stability of zinc binding to the fully reduced hMT ($K_d = 1.4 \times 10^{-13}$ M). However, the zinc-thiolate clusters within the hMT are highly reactive and can be oxidized even by mild cellular oxidants including GSSG, causing release of zinc: A combination of GSH and GSSG enhances zinc transfer from hMT to a zinc acceptor (41). These studies elegantly demostrated not only redox-regulated thiol–disulfide exchange within a zinc–thiolate cluster, but also that this exchange involves cysteine residues that bind zinc. The studies suggest unique roles for zinc and sulfur in allowing thiol–disulfide exchange within a physiological range of redox change. Although hMT has a low redox potential (<-366 mV), it seems likely that the zinc–cysteinyl bonds within some zinc finger transcription factors will also undergo thiol–disulfide exchange reactions with GSH/GSSG. Redox-dependent release of zinc has recently been demonstrated for the zinc finger motifs in protein kinase C (26, 47).

DIRECT EVIDENCE FOR PHYSIOLOGICALLY RELEVANT ZINC FINGER OXIDATION

Oxidation of ER zinc finger in breast cancer

Steroid hormones are lipophilic molecules derived from cholesterol and synthesized in the adrenal cortex, testis, ovary, and placenta (for review, see 11). Because of their lipophilic nature, they diffuse unassisted from the blood to the cell cytoplasm where they bind to specific nuclear hormone receptors (NHRs). NHRs are intracellular transcription factors that are usually complexed to chaperones, such as heat-shock proteins, and are activated by the high-affinity binding of hormone ligand. Binding of the ligand usually results in nuclear translocation of the hormone–receptor complex and subsequent binding to specific DNA sequences termed hormone response elements (HREs) that regulate the expression of the target genes. These complexes can regulate the activity of many genes and modulate numerous responses in many cell types. ER, GR, and the retinoic acid receptor are all members of this NHR superfamily, and there is now strong evidence that each of these NHRs is under physiological redox control (17, 21, 30, 54, 57, 67). ER regulation by oxidative stress, for example, may contribute to the metastatic phenotype of some breast cancer cells.

The ER, like other NHRs, has two zinc fingers of the Cys_4 class that are essential for DNA binding to the HRE, as well as for protein–protein interactions (54, 88). ER isolated from approximately one third of untreated ER-positive breast tumors is unable to bind to its cognate HRE (54). Tumors containing the abnormal ERs have reduced responsiveness to estrogen and tamoxifen therapy, and the disease prognosis is poorer for these cancers compared with tumors that have normal ER DNA-binding capacity (54). The defective ER DNA-binding activity in these cells can be significantly reversed by treating cell extracts with DTT (30). The purified DNA-binding domain of ER, which contains the zinc fingers, is highly sensitive to oxidation or alkylation *in vitro*. Both treatments target the thiol groups in the zinc fingers and prevent DNA binding which, in the case of ox-

idation, can be reversed by reducing agents. Treatment of breast cancer cells possessing normal ER-binding activity with oxidizing agents results in selective ER oxidation, reduced binding capacity, and reduced expression of ER-responsive genes. These effects are similar to the GR responses discussed earlier.

The second zinc finger of ER, which is involved in dimerization at the HRE-binding site, has been identified as the likely target for oxidation *in vitro* and *in vivo* (88). The first DNA-binding zinc finger is protected from oxidation when zinc is bound, but the second finger, which is more flexible, remains fully sensitive to oxidation even within the zinc–DNA complex. Loss of ER DNA binding is associated with intraprotein disulfides involving the second zinc finger (88). Oxidation may result from high levels of reactive oxygen present in the tumor microenvironments, possibly generated by repetitive cycles of ischemia and reperfusion (54, 88).

Age-related oxidation of Sp1 zinc fingers

As discussed earlier, Sp1 is a member of an extended family of DNA-binding proteins that have three zinc finger motifs and bind to GC-rich DNA (20, 43). Sp1 is ubiquitously expressed in mammalian tissues, although with widely different tissue abundance (70). It binds with different affinities to DNA sites that have variations of the preferred consensus sequence, and it contributes to the transcriptional regulation of numerous genes (25, 45, 46, 49). Sp1 factors have been shown to play critical roles in the transcriptional regulation of a class of genes that do not contain classical TATA or CAAT box elements in their proximal promoters (90). These include a number of so-called housekeeping genes, such as glycolytic enzymes, DHFR, thymidylate synthase, and adenine deaminase (83), as well as some non-housekeeping genes (15, 23, 93), erythroid-specific genes (18, 60), and the stress-related MT (8).

Ammendola *et al*. first reported that the binding activity of Sp1, but not the amount of immunoreactive protein, was dramatically reduced in the brain and liver of 30-month-old rats compared with younger animals (6, 7). They found no age-related changes in the binding of other factors including nuclear factor 1 (CTF/NF1). In these studies, the reduced Sp1 binding activity was fully restored when nuclear extracts from aged rat brain or liver were treated with DTT. Conversely, Sp1 binding activity from young rat brains was reversibly blocked by oxidizing agents, supporting the probable involvement of oxidation in age-associated Sp1 inactivation. Similar results were reported by Helenius *et al*., who found that Sp1 binding activity, but not that of either AP1 or NF-κB, was significantly reduced in aged rat hearts (31). These changes of Sp1 function have been correlated with age-related loss of transcriptional activity of a number of genes, including the H ferritin gene, transferrin (3, 43), and the androgen receptor (82). Considering the magnitude of the effects reported and the number of genes regulated by Sp1, it is surprising that more age-related gene casualties have not been reported.

The effects of aging on Sp1 binding may be analogous to the accumulation of oxidized ER in breast cancers. Both appear to involve selective oxidation of zinc finger thiols, and both involve physiological conditions that are associated with elevated oxidative stress. ER and Sp1 belong to separate classes of zinc fingers, Cys_4 and Cys_2His_2, respectively, but both are reversibly oxidized by oxidizing agents *in vitro*. Although the second zinc finger of ER has been identified as the redox-sensitive site, it has not been determined whether any one of the three Sp1 zinc fingers is selectively oxidized. The first Sp1 zinc finger has four charged amino acids flanking the cysteines and is probably the most reactive finger of the group with greatest probability for disulfide exchange.

SUMMARY AND CONCLUSIONS

Evidence from multiple sources supports the conclusion that zinc finger transcription factors are susceptible to reversible oxidation. In particular, the DNA binding and transcriptional activating functions of Sp1, Egr-1, GR, and ER are inhibited by oxidizing agents and reactivated by reducing agents. These factors are also irreversibly inactivated by treatment with *N*-ethylmaleimide and reversibly inactivated by

reaction with thionein or mithramycin, both of which displace zinc (14, 68, 94). Although detailed biochemical analyses have not been done in all cases, the accumulated data support the direct involvement of zinc-coordinated sulfhydryl groups in these redox exchanges. The very existence of oxidoreductase enzyme systems, such as Ref-1 and thioredoxin, that can specifically target zinc finger sequences underscores the physiological importance of this redox regulation. Direct evidence of thiol–disulfide interchange and zinc displacement within a physiological range of redox change has been described for zinc–thiolate clusters in hMT and the zinc finger domains of protein kinase C. In all cases, the oxidative stress is probably transmitted to the target thiol groups by thiol/disulfide reactions with GSH and GSSG. High concentrations of GSH will normally stabilize zinc thiolates, and oxidation may only be evident under conditions of heightened oxidative stress when the GSH: GSSG ratio falls.

De novo synthesized zinc finger proteins without bound metals and related secondary folding may be more sensitive to oxidation than the final metal-associated conformations. Binding of zinc fingers to DNA in most instances appears to confer further protection, probably by insulating the zinc–thiol groups and sequestering factors in the nucleus. There may be a wide range of susceptibility to oxidation within zinc finger classes, determined by the redox potential of the thiols, secondary protein folding, and physical location. For example, unliganded Cys_4 hormone receptors are sequestered in the cell cytoplasm and may be more accessible to oxidation than their DNA-bound counterparts in the nucleus. Sp1 factors are usually tightly DNA-bound and should be resistant to oxidation. The increased levels of oxidized Sp1 observed during aging may result from an increased rate of oxidation of *de novo* synthesized protein without a compensatory increase in protein production. The GATA transcription factors, which like the NHRs comprise a large family of Cys_4 zinc finger proteins, have not been reported to be directly responsive to redox change. Therefore, whereas all zinc finger thiolates are theoretically susceptible to oxidation, only a small fraction of them may be physiological targets. Secondary structures, physical location, and targeting by oxido-reductase enzymes may play determining roles in this susceptibility.

Because of the strong and often opposite selective pressures exerted by oxygen and oxygen-derived free radicals, redox mechanisms occupy elevated positions in the cell's regulatory hierarchy. Cellular homeostasis appears to require the maintenance of a strict equilibrium between reactive oxygen and the quenching systems, suggesting that reactive oxygen is required for normal functions and can play both negative and positive roles in cell signaling. Oxidation and inactivation of ER and Sp1 appear on the surface to be of entirely negative survival value. Oxidation of hMTs with release of zinc, redox-mediated fine tuning of protein kinase activity, and the activation of signaling cascades may be examples of positive roles for reactive oxygen that transcend a simple counterresponse to stress.

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ABBREVIATIONS

AP1, activator protein-1; BSO, L-buthionine-[*S*,*R*]-sulfoximine; DHFR, dihydrofolate reductase; DTT, dithiothreitol; Egr-1, early growth response factor-1; ER, estrogen receptor; GR, glucocortocoid receptor; GSH, reduced glutathione; GSSG, oxidized glutathione; hMT, human metallothionein; H_2O_2, hydrogen peroxide; HRE, hormone response element; MT, metallothionein; NF-κB, nuclear factor-κB; NHR nuclear hormone receptor; Ref-1, redox factor-1; SOD, superoxide dismutase; SRF, serum response factor; TFIIIA, transcription factor IIIA.

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