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COMPREHENSIVE INVITED REVIEW

Redox Control of Protein–DNA Interactions: From Molecular Mechanisms to Significance in Signal Transduction, Gene Expression, and DNA Replication

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Abstract

Protein–DNA interactions play a key role in the regulation of major cellular metabolic pathways, including gene expression, genome replication, and genomic stability. They are mediated through the interactions of regulatory proteins with their specific DNA-binding sites at promoters, enhancers, and replication origins in the genome. Redox signaling regulates these protein–DNA interactions using reactive oxygen species and reactive nitrogen species that interact with cysteine residues at target proteins and their regulators. This review describes the redox-mediated regulation of several master regulators of gene expression that control the induction and suppression of hundreds of genes in the genome, regulating multiple metabolic pathways, which are involved in cell growth, development, differentiation, and survival, as well as in the function of the immune system and cellular response to intracellular and extracellular stimuli. It also discusses the role of redox signaling in protein–DNA interactions that regulate DNA replication. Specificity of redox regulation is discussed, as well as the mechanisms providing several levels of redox-mediated regulation, from direct control of DNA-binding domains through the indirect control, mediated by release of negative regulators, regulation of redox-sensitive protein kinases, intracellular trafficking, and chromatin remodeling. *Antioxid. Redox Signal*. 13, 1429–1476.

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I. Introduction

Protein–DNA interactions are responsible for the regulation of key biological functions, including transcription, replication, repair, and recombination. Specificity of these interactions is determined by the recognition of unique DNA sequences in the genome by their counterpart *trans*-acting DNA-binding proteins. These proteins are often grouped into classes based on the structural motif they use in the interactions with their DNA-binding site, including the helix-turn-helix (HTH), homeodomain, various types of zinc finger structures, steroid receptors, leucine zipper, helix-loop-helix, and β-sheets elements. Interaction of regulatory DNA-binding proteins with their specific target sites in the genome is often preceded by their nonspecific binding to DNA, an important intermediate step in the process of sequence-specific recognition and binding, which significantly accelerates the search for the specific target site in the genome [reviewed in Refs. (148, 262, 356)].

One of the largest and most diverse classes of DNA-binding proteins is the group of transcription factors. These proteins regulate gene expression and thereby control multiple processes in cell metabolism, growth, development, immune response, and cell survival, by binding to specific promoters and enhancers in the genome and activating a series of genes in response to cellular and external stimuli. Another important group of sequence-specific DNA-binding proteins includes the replication initiator proteins, the origin-binding proteins and the origin recognition complexes, whose specific interactions with replication origins in the genome trigger the initiation of DNA replication, in response to signals transmitted by the cell cycle control.

A. Signal transduction using reactive oxygen species and reactive nitrogen species

Cellular control of interactions of regulatory proteins with their specific binding sites in the genome is largely mediated

through the modulation of these proteins by a large array of posttranslational modifications, including phosphorylation, acetylation, carboxylation, glycosylation, hydroxylation, methylation, and ubiquitination, which are triggered by signals transmitted in response to physiological stimuli. The consequent structural modifications elicited in the target proteins can modulate their DNA-binding properties, catalytic activity, stability, or their intracellular trafficking. Within this growing list of posttranslational modifications, data accumulated during the last three decades clearly demonstrate the significance of oxidation–reduction (redox) of proteins, in the regulation of major cellular processes. Redox regulation is mediated through the action of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The term ROS encompasses many species, including superoxide (O_2^-), hydroxyl radical (HO•), and hydrogen peroxide (H_2O_2). RNS includes species such as nitric oxide (NO•) and peroxynitrite. Several of these ROS and RNS were found to play a major role in the regulation of protein–DNA interactions. Intracellular ROS and RNS are contributed by both exogenous and endogenous sources. At high concentrations, free radicals and nonradical reactive species are hazardous for the cells, which under physiological conditions maintain redox balance through generation and elimination of ROS and RNS. At moderate concentrations, ROS and RNS play an important role as regulatory mediators in signaling processes (150, 341).

Different mechanisms for redox sensing have been observed in the various redox-regulated proteins. While in many of them, reactive cysteine(s) residues function as redox sensors, others use the redox-sensitive metal iron, coordinated in Fe–S clusters, as a sensor [reviewed in Ref. (161)]. Many redox-regulated proteins use their thiol-coordinated zinc sites as redox switches. In these cases, redox sensing occurs at the coordinating cysteine residues and not through the metal ion. Proximity of the metal ion maintains the thiols as deprotonated, highly reactive thiolate anion. Hence, upon exposure to ROS, these reactive cysteines are readily oxidized, and the zinc ion is released, which results in the disruption of the conformation of the zinc site, causing a conformational change in the protein, that leads to its functional modulation [reviewed in Refs. (17, 130)].

B. The specificity and transmission of a redox signal

1. Factors affecting redox signaling specificity. Although the use of ROS and RNS in redox-mediated signaling pathways had been widely documented, it remained controversial. This controversy stems from the fact that redox signaling, which is mediated by small reactive ROS/RNS, does not fit the current concept of specific recognition, which is based on complementarity (by parameters such as of shape, charge, or hydrophobicity), leading to the formation of a transient noncovalent binding, which often occurs in recognition between two macromolecules. Signaling by ROS and RNS, on the other hand, occurs through chemical reactions with specific atoms of target proteins; thus, recognition is at the sub-molecular, atomic level, and consequently has the potential for recognition of many ROS/RNS-specific receptors (249). Nevertheless, in most redox-regulated proteins studied, including the systems discussed in this review, the targeting of specific reactive groups within specific targeted proteins is evident. However, the general principles underlying the specificity in redox signaling are not yet fully understood. Questions regarding the mechanisms that determine the specificity of redox signaling pathways have recently been discussed on several occasions (71, 97, 249, 369).

One factor that can limit the number of potential targets and thereby may provide some level of specificity, under low concentrations of ROS/RNS, relies on the reactivity of the interacting groups in the targeted proteins. It is widely accepted that oxidation and reduction of thiol proteins is the major mechanism by which reactive ROS and RNS act in transmitting the redox signal. Most redox-regulated proteins studied share the presence of one or more highly reactive cysteine residues, which become reversibly modified upon exposure to ROS/RNS. The nucleophilic reactivity of the cysteine thiols in these proteins was found to be strongly dependent on the surrounding chemical microenvironment and its effects on the thiols dissociation constant. Cysteine thiol's pKa is decreased when located proximal to positively charged groups of basic amino acids, aromatic amino acids, or metal centers that can interact with the thiol group and facilitate its deprotonation into the more reactive thiolate anion. This difference in reactivities between cysteine residues provides specificity, by virtue of the preferential modification of these residues by reactive species [recently reviewed in Refs. (97, 130, 292, 369)].

In addition to the reactivity of specific cysteine residues of target proteins, other levels of specificity could be provided by the type of the reactive species, the intracellular site in which they are generated, and their *trans*-membrane permeability. The fact that different ROS and RNS have a wide range of different reactivities with thiols may affect the choice of target and reactive species. In addition, the subcellular compartmentalization of the target and the proximity of the source of the reactive species may also play a role in specificity [reviewed in Refs. (68, 130, 292, 369)]. Membrane permeability of the reactive species is another constraint that should be considered, as reactive species permeability through biomembranes vary considerably, from that of H_2O_2, which is less limited by membrane permeability, to that of O_2^-, which displays very limited permeability through lipid membranes and can use ion channels for this purpose. However, although biological membrane are almost impermeable to O_2^-, this species can be protonated to hydroperoxyl radical at low pH ($pK_a = 4.8$), and the uncharged form of the molecule is membrane permeable (112, 246).

Overall, activity of some reactive species may be confined to subcellular compartment in which they were generated [recently reviewed in Ref. (369)]. The question of membrane permeability may be further complicated by the effect of the reactive species on membrane permeability, as is demonstrated in the case of H_2O_2. In contrast to the widely accepted belief that H_2O_2 diffuses freely through biomembranes, recent studies have shown (35) that H_2O_2 diffusion through the plasma membrane results in a decrease in the rate of consumption of extracellular H_2O_2. Further studies have shown that these changes are accompanied by an increase in fluorescence anisotropy, indicating that membrane fluidity is decreased (96). It has further been shown that the adaptation of *Saccharomyces cerevisiae* to H_2O_2 exposure results in significant changes in the lipid profile of the plasma membrane, and in expression of genes coding for enzymes involved in lipid biosynthesis (273).

2. Transmission of a signal via redox sensors. Oxidant-specific sensors regulate the concentration of peroxides in bacterial cells by measuring their level and regulate expression of oxidant-scavenger genes according to this level. These sensors detect very low levels of H_2O_2, preventing the cellular damage induced by oxidative stress. *Escherichia coli* OxyR (389) carries out both the H_2O_2 sensing function and gene expression regulatory function. Unlike in bacteria, it was found that in the yeast Orp1–Yap system the H_2O_2 sensor, Orp1, and the downstream transcriptional regulator, Yap1, are separated components of the system [recently reviewed in Ref. (71)]. On the basis of observations in both the bacterial and eukaryotic systems, Toledano *et al.*, (333) have proposed that considering the specificity of the H_2O_2-sensing phenomenon, only very limited set of cysteine residues in the cell may interact with the oxidant and will be directly affected by it. These thiol-based "peroxide receptors" or "sensors" that react with H_2O_2 form a hierarchy in the oxidation of thiols in other proteins by interacting with them (333). This concept was similarly introduced in metazoa in a study of redox regulation of the phosphatase and tensin homolog protein and protein tyrosine phosphatases in H_2O_2-mediated cell signaling, by Cho *et al.* (58). These investigators proposed that oxidation might also be mediated by a thiol peroxidase (like GPX3), functioning as an H_2O_2 sensor and transducer of the hydroperoxide signal. The model proposing that oxidation of most target proteins is not conducted directly, but rather mediated through the oxidation of a subset of very reactive thiols in sensor proteins, which facilitate the oxidation of other target proteins through selective protein–protein interactions, was discussed recently by Winterbourn and Hampton (369). By modeling cellular thiols as targets for H_2O_2 in mammalian cells, these investigators suggested that, of the proteins considered, the 2-Cys peroxiredoxins (PRX) are favored as targets and may therefore be involved in transmission of the redox signal, whereas other thiol proteins with lower reactivity would be oxidized by an indirect mechanism. A redox signal is transmitted from the site of ROS generation, mainly in the mitochondria, through the sensor protein to its final destination, which may reside, as in the case of the transcription and replication factors, in the nucleus. The nucleus is generally considered to provide a reducing environment. Thus, the different redox states within these different subcellular compartments may present an additional constraint, in the transmission of redox signal. In a recent study, Markovic *et al.* (221) have revealed that the nuclear redox state is not static but fluctuates in a cell-cycle-dependent manner. Several redox pairs, including the nicotinamide adenine dinucleotide (NADH/NAD+), the nicotinamide adenine dinucleotide phosphate (NADPH/NADP+) and the glutathione/glutathione disulfide (GSH/SSSG) pairs, are known to affect nuclear redox state [recently discussed in Ref. (207)]. GSH is the most abundant nonprotein thiol in mammalian cells and is found within the cell in both its reduced and oxidized forms. Changes in the GSH/GSSG ratio indicate the disturbance of the delicate balance in the intracellular redox state. Markovic *et al.* (221) have shown that nuclear GSH distribution undergoes periodical changes with the progress of the cell cycle. These investigators have measured a significant increase in GSH concentration in the nucleus during $S + G_2/M$ phase of the cell cycle, with concentrations of more than fourfold higher than those measured in the cytoplasm, whereas approximately equal nuclear and cytoplasmic concentrations of GSH were measured during G_0/G_1. Thus, although the nucleus is generally considered a reducing compartment, its reducing capacity fluctuates and this fluctuation may have a considerable effect on the transduction of a redox signal from the ROS generating cytoplasmic compartment to a nuclear protein acceptor.

This review discusses the redox regulation of several master transcriptional regulators, which control cell growth, development, and survival and its defense, in confronting external and internal stresses. It also discusses redox-regulated proteins that function in DNA replication, a fundamental cellular process in which the role of redox regulation has yet to be established. The review discusses a broad scope of redox-mediated protein–DNA interactions and describe the series of events that precede and regulate DNA binding, which culminate in the interactions of the regulatory protein with its specific binding site. It focuses on eukaryotic systems, mainly mammalian cells. Bacterial and yeast systems have been recently discussed elsewhere (71, 335). The implications of many of the protein–DNA interactions described in this review on the physiology of mammalian cells and their survival have been the subject of a recent review (341).

II. Redox Regulation of Transcriptional Control

Redox regulation of gene expression is a strategy that has been conserved in evolution from bacteria to mammalian cells in which ROS and RNS that are produced in the cell are used in signal transduction to reversibly affect discrete reactive amino acid residues, mainly cysteine's thiols, in transcription factors. The presence of redox-sensitive reactive cysteine residues in their DNA-binding domains and the redox sensitivity of these transcription factors (115, 342) raised the suggestions in early studies that the direct redox modification of proteins is a major mechanism underlying redox signaling pathways. More recent studies suggest that gene expression is regulated by additional, indirect redox-controlled mechanisms. It has been found that eukaryotic gene expression in response to redox signaling is mediated not only through the direct redox effect on the DNA-binding site of transcription factors. Instead, the activity of several DNA-binding proteins, including major transcription factors, has been found to be regulated by their redox-sensitive association with interacting proteins, which most often function as their negative regulators, including the pairs of nuclear factor kappa B (NF-κB) and inhibitor kappa B (IκB), nuclear factor E2-related factor 2 (Nrf2), and the Kelch-like erythroid-cell-derived protein with cap 'n' collar (CNC) homology (ECH)-associated protein 1 (Keap1), Jun amino terminal kinase (JNK), and GSH-S-transferase (GST), p53, and the mouse double-minute-2 protein (Mdm2), and the apoptosis signal-regulating kinase 1 (ASK1) and thioredoxin (TRX). Other studies revealed that chromatin remodeling provides another major redox-sensitive regulatory mechanism that functions through the action of the histone deacetylases (HDAC), whose redox-sensitivity provides an indirect level of regulation of transcription factors' interactions with their target site in the genome, by affecting the local unwinding of DNA in the chromatin structure and thereby the accessibility of the transcription machinery to the targeted gene (285). Another level of regulation by an indirect mechanism is provided by the redox regulation of phosphorylation of transcription factors through redox-sensitive protein kinases, such as the mitogen-activated protein (MAP)

kinases (MAPK) and protein kinase C (PKC) [e.g., Ref. (267)]. Yet, additional level of redox-mediated regulation is provided by the activator protein (AP) endonuclease 1/redox factor 1 (APE1/Ref-1) protein that affects the redox state of reactive cysteine residues and thereby induces the DNA-binding activity of several transcription factors such as AP-1, hypoxia-inducible transcription factor (HIF), NF-κB, p53, and others, through a redox cycle in which TRX restores the reduced form of APE1/Ref-1, as well as by acting as a redox chaperone (12, 328). The transcription factors described below are master regulators of gene expression, responsible for the induction of numerous metabolic pathways, which they induce or repress in response to physiological stimuli. They form intricate transcriptional control networks in the cell, which interact with each other and affect the outcome of the networks' regulatory activities.

A. Activator protein 1

1. AP-1 structure and function. The AP-1 family of transcription factors consists of several protein subfamilies containing a basic leucine zipper (bZIP) domain. Members of the AP-1 family are involved in the regulation of central cellular processes, including cell proliferation and survival, growth, differentiation, apoptosis, cell migration, and transformation. AP-1 proteins can function as antioncogenic factors, by inducing apoptosis, or as oncogenic factors, by signaling cell survival [reviewed in Refs. (86, 123, 155, 216, 308, 353, 354, 383, 384)]. Typical to bZIP proteins (Fig. 1), AP-1 transcription factors contain a basic domain, involved in sequence-specific DNA binding, and a leucine zipper domain, consisting of a coiled-coil structure with repeats of leucines clusters, which are involved in protein dimerization [for a recent review on bZIP proteins see Ref. (216)]. AP-1 proteins bind a specific sequence motif in the DNA, designated AP-1/TPA (TPA, 12-*O*-tetradecanoylphorbol-13-acetate) responsive element (AP-1/TRE: TGA(C/G)TCA). The AP-1 family includes four subfamilies of transcription factors: Jun (c-Jun, JunB, and JunD); Fos (c-Fos, FosB, Fra-1, and Fra-2); musculoaponeurotic fibrosarcoma (Maf), including c-Maf, MafB, MafA, MafG/F/K, and Nrl; and activating transcription factor (ATF), including ATF2, LRF1/ATF3, B-ATF, JDP1, and JDP2. In yeast, a Jun homolog, named general control nondepressible 4 protein (Gcn4p) recognizes the same AP-1/TRE DNA motif. AP-1 proteins bind the AP-1/TRE motif in the promoter and enhancer regions of mammalian genes, as dimers.

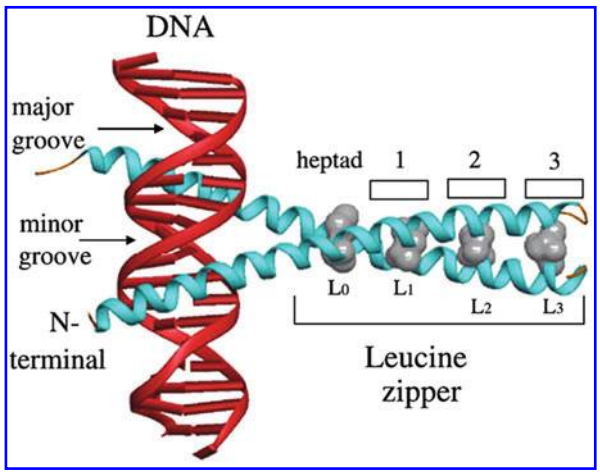

FIG. 1. A model of GCN4 B-ZIP dimer bound to double-stranded DNA. Each monomer is a long bipartite α-helix. The N-terminal half contains basic amino acid residues that interact with the major groove of DNA in a sequence-specific manner. The C-terminal half is a leucine zipper—an amphipathic α-helix that dimerizes to generate a parallel coiled-coil. The first three heptads of the leucine zipper are numbered. The DNA is in *red*, while the basic leucine zipper α-helices in *blue* and the leucines of their heptad repeats in *gray*. Reprinted with permission from Elsevier (355), Copyright (2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Jun and Fos are the major AP-1 proteins functioning in mammalian cells. Their identification as the cellular homologs of retroviral oncoproteins v-Jun and v-Fos, as well as their induction by growth factors and tumor promoters, linked AP-1 to cellular growth control and neoplastic transformation (123). Jun and Fos differ in their requirements for the binding of DNA. Both Jun homodimers and its heterodimers have DNA-binding capacity. Fos binds DNA only in a heterodimer with Jun. There is a high degree of structural homology between the different proteins of the Jun and Fos subfamilies. However, the distinct AP-1 dimers display significant differences in their DNA-binding and transactivation activities (308). AP-1 activities are modulated through the differential expression of its individual components, which determines its dimer composition. The significance of AP-1 composition in tumorigenesis has been shown by several approaches, including the use of AP-1 monomers that were joined by a flexible polypeptide tether, or using dimer-specific mutants of AP-1 proteins, in which manipulation of the leucine zipper domain allows the formation of specific dimers. These studies indicated that various AP-1 dimer combinations are required at different stages of tumorigenesis and some dimers suppress tumorigenesis (86, 348).

2. Regulation of AP-1 activity. Multiple different stimuli, including cytokine, growth factors, neurotransmitters, hormones, bacterial and viral infections, stress signals, and oncogenic stimuli induce AP-1 (Fig. 2). Its activity can be regulated at the levels of transcription of the genes encoding AP-1 subunits, mRNA stability, translation of mRNA, protein turnover, and posttranslational modification (86, 156, 354). Additional layers of regulation are applied by specific interactions between AP-1 proteins and other transcription factors and cofactors that modulate AP-1 transcriptional activity (123), as well as by chromatin remodeling. Members of the AP-1 group are expressed and regulated in a cell-type-specific manner; hence, different cells may contain a complex mixture of AP-1 dimers with discrete functions (123, 308, 383).

Posttranslational modification of AP-1 proteins, especially phosphorylation of Fos and Jun, has been studied extensively, demonstrating that all three classes of the MAPK pathways, which include the extracellular-signal-regulated kinases (ERKs), JNKs, and the p38 kinases, play a major role in AP-1 activation, through phosphorylation of specific serine and threonine residues (155, 384). Binding of a serum-responsive element (SRE) by the serum-responsive factor mediates

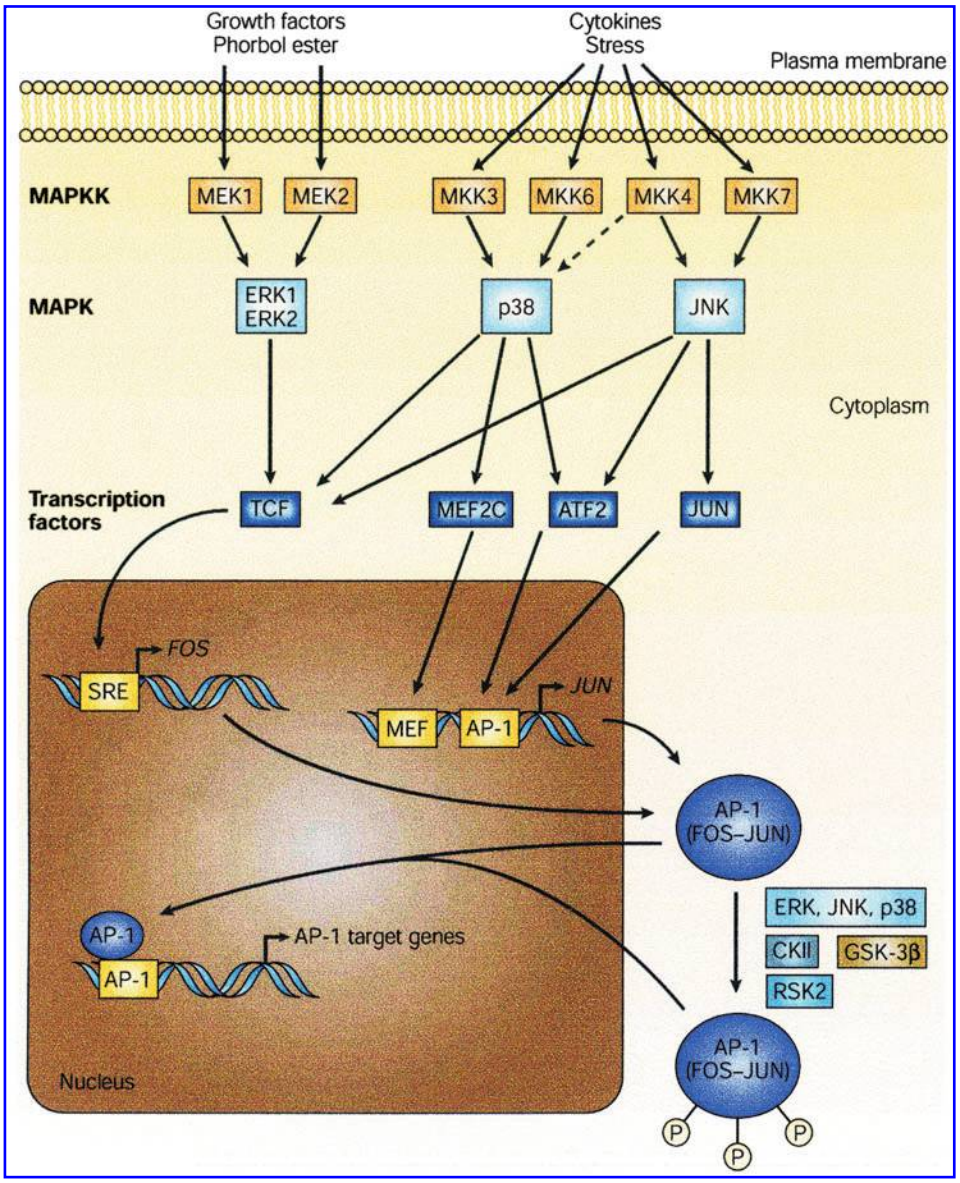

FIG. 2. Activation of AP-1. AP-1 activity is stimulated by a complex network of signaling pathways that involves external signals, such as growth factors, cytokines, oxidative and other stresses, and MAPKs of the ERK, p38, and the JNK families. MAPKs activate various transcription factors (TCFs, MEF2C, ATF2, and JUN) that induce the transcription of *FOS* and *JUN* genes, thereby increasing the number of AP-1 complexes and activating AP-1 target genes. Posttranslational phosphorylation by various kinases regulates AP-1 activity, which includes its transactivating potential, DNA-binding capacity, and the stability of AP-1 components. AP-1, activator protein 1; ATF2, activating transcription factor 2; CK II, casein kinase II; ERK, extracellular-signal-regulated kinase; GSK-3β, glycogen synthase kinase-3β; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEF2C, myocyte-enhancer factor 2C; RSK2, ribosomal S6 kinase 2; SRE, serum-response element; TCFs, ternary-complex factors. Reprinted with permission from Macmillan Publishers Ltd. (86), Copyright 2003. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

the induction of c-Fos by stimuli that activate MAPK by recruitment of a ternary complex factor (TCF), activated by ERK MAP kinases. Jun is expressed constitutively, at low levels, in many cell types and its expression is enhanced significantly in response to various stimuli, including growth factors, cytokines, and UV irradiation. With the accumulation of AP-1 complexes and activation of JNK and p38 MAP kinases, JNKs phosphorylate two regulatory functional residues within the Jun N-terminal domain, and JNKs and p38 phosphorylate ATF-2, which stimulates the transactivation potential of these transcription factors. Glycogen synthase kinase-3 (βGSK-3β) and ribosomal S6 kinase 2 (RSK-2) also phosphorylate Fos and Jun proteins, regulating their transactivation potential and DNA-binding activity [reviewed in Refs. (86, 308)].

Phosphorylation of c-Jun and c-Fos enhances the association between c-Jun/c-Fos and the transcriptional coactivator cAMP response element-binding protein (CREB), p300/CBP, which does not bind to DNA *per se*, but upon its recruitment, acts as a bridge between the sequence-specific transcription factors and the general transcriptional machinery, to promote transcriptional activation of a target gene. As described below, CBP/p300 also possesses histone acetyl transferase (HAT) activity that, through chromatin remodeling, promotes the accessibility of the transcription factor to the specific binding sequence at the promoter or enhancer of target genes (216, 217). Phosphorylation of Fos and Jun has also been shown to alter their interactions with other transcription factors, affecting DNA binding and dimerization (123, 216), and to be associated with both the activation and inhibition of DNA binding and gene transcription. It has been found that different protein kinases can distinguish between the distinct monomer, homodimer, and heterodimer forms of Fos and Jun proteins and between DNA-bound and unbound proteins. Phosphorylation of residues at the c-Jun DNA-binding domain by GSK-3β results in inhibition of Jun DNA-binding activity, whereas dephosphorylation of these sites, as a result of the activation of PKC, reactivates Jun capacity to bind DNA (1, 32), indicating a complex relationship between AP-1 phosphorylation and function.

In addition to transcriptional and posttranslational control mechanisms, translational regulation of the mRNAs encoding the AP-1 subunits contributes to the balanced production of AP-1 proteins and thereby to their intracellular abundance [for a recent review of AP-1 translational regulation see Ref. (354)]. Both cap-dependent and cap-independent mechanisms were observed. Translational regulation of ATF4/5 (391) and JunD (313) mRNAs is mediated via mechanisms that are all dependent on upstream open reading frames (upORFs) in the 5′-leader sequence. c-Fos is an unstable transcription factor with a rapid nuclear turnover. Efficiency of its mRNA translation was found to decrease by specific miRNAs (354). Translation of JunB mRNA is controlled via mTOR (mammalian target of rapamycin) found in 5′-TOP mRNAs, which contain oligo-pyrimidine tracts after the 5′-CAP site. Pharmacological inhibition of mTOR resulted in downregulated JunB protein levels, by shifting JunB mRNA translation from large polysomes into monosomes and ribonucleic particles (RNPs) (318). It was found that c-Jun mRNA could be regulated by both CAP-dependent and CAP-independent translational control mechanisms, via an internal ribosome entry segments (IRES) (302), whereas it was indicated that IRES elements do not play a role in the translational regulation of JunD mRNA (313).

3. The redox regulation of AP-1 function. Overall, Ap-1 transcriptional activity is controlled by ROS and RNS at several levels of its regulation. This includes the regulation of expression of the genes encoding AP-1 subunits, the interactions of the AP-1 proteins with their DNA-binding sites at the promoter or enhancer of the target genes, their interactions with coactivators, as well as at the level of chromatin remodeling, through the promotion of the action of HAT and inhibition of HDAC [reviewed in Refs. (224, 310, 341)]; this issue is further discussed in section II.B.6.

As described above, the three groups of MAP kinases, ERK, JNK, and p38, which were shown to be activated in response to the intracellular redox state and oxidative stress, are involved in the regulation of AP-1. Oxidative stress leads to ERK1/2 activation. Growth factor receptors play an important role in mediating this process (181). JNK and p38, the stress-activated protein kinases (SAPKs), are involved in signaling for survival, or apoptosis, in response to oxidative stress. They are activated by ASK-1, which serves as a redox sensor for activation of the stress-activated protein kinase cascade, by phosphorylation of their MAPK kinases (MKKs) [reviewed in Refs. (224, 321)].

a. Redox regulation of ASK-1 function. ASK1 contains a reduction/oxidation active site, consisting of two cysteine residues within the conserved sequence Trp-Cys-Gly-Pro-Cys-Lys, providing the sulfhydryl groups involved in TRX reducing activity and ASK-1 inhibition. TRX was found to be associated with ASK-1 N-terminal domain in nonstressed cells (293). ASK-1 activity depends on the redox state of TRX, which only in its reduced form is capable of binding ASK-1. TRX binding to ASK-1 blocks its kinase activity. In the presence of ROS, upon oxidation of TRX reactive thiol groups, ASK1 dissociates from TRX, resulting in its activation. ROS-dependent dissociation of TRX from ASK-1 was impeded by treatment of cells with antioxidants. TRX inhibition resulted in activation of endogenous ASK-1, indicating that TRX is a physiological inhibitor of ASK-1 and that under basal unstressed conditions ASK-1 is sequestered by TRX in an inactive form (7, 321). In a study of ASK1 regulation by H_2O_2, Nadeau *et al.* reported that exposure of cells to H_2O_2 (0.1–1 mM) caused rapid oxidation of ASK-1, leading to its multimerization, through the formation of disulfide-linked high-molecular multimeric forms, which is essential for full activation of JNK. Oxidized ASK-1 is rapidly reduced by TRX that becomes covalently associated with ASK-1, preventing its oxidation, which impairs the full activation of JNK and the induction of apoptosis (248). S-nitrosylation was found to affect the regulation of apoptosis through its effect on TRX–ASK-1 interactions and further interactions with TRX and ASK1 [reviewed in Ref. (122)] (Fig. 3).

Studies on the role of glutaredoxin (GRX) in oxidative stress-induced signaling and cytotoxicity in glucose-deprived human cancer cell (316) revealed that similarly to TRX, GRX binds to ASK-1 and thereby functions as a negative regulator. Upon glucose deprivation, the increased levels of ROS lead to GRX oxidation and consequently to its dissociation from ASK-1 and ASK-1 activation. Cysteine residues at the GRX active site were required for dissociation of GRX from ASK-1 during glucose deprivation. Cells overexpressing GRX were resistant to glucose deprivation-induced ROS and showed decreased activation of JNK. The redox-sensitive GRX–ASK-1 interaction was found to be regulated in a GSH-dependent manner by H_2O_2. Overexpression of GRX or catalase activity inhibits the activation of ASK-1 signaling during glucose deprivation, suggesting that dissociation of GRX from ASK1 activates ASK1 signaling pathway.

Several other mechanisms have been proposed to function in the control of the ROS-mediated ASK-1 activation. The binding of the phosphoserine/phosphothreonine-binding protein 14-3-3 to ASK-1 through phosphorylated Ser-967 suppresses ASK-1-induced apoptosis. Treatment of cells with H_2O_2 (0.5–5.0 mM) triggers dephosphorylation of Ser-967, resulting in dissociation of the ASK-1 complex with 14-3-3, with concomitant increase in ASK-1 catalytic activity and its mediated activation of JNK and p38 pathways (106). In addition, protein phosphatase 5 (PP5) has also been found to function as ROS-dependent ASK-1 inhibitor. In response to ROS, PP5 binds to the activated ASK-1 and directly dephosphorylates an essential phospho-threonine residue within the kinase domain of Ask-1, inhibiting the H_2O_2-induced activation and ASK-1-dependent apoptosis (242). It has also been reported that in H_2O_2 (0.1–4.0 mM)-treated endothelial cells, phosphorylated protein kinase D binds to ASK-1, a process that is critical for induction of JNK activation by H_2O_2, but not by tumor necrosis factor-α (TNFα) (387).

Several reports have described the involvement of GSTs π and μ in the regulation of MAPK-dependent apoptotic pathway, via protein–protein interactions with JNK-1 and ASK-1 [reviewed in Ref. (340)]. GST suppresses ASK1 in a way similar to that found in the case of TRX, in which association between the two enzymes occurs at basal nonstressed redox conditions and their dissociation is induced by ROS, leading to Ask-1 activation (7, 340). GST–JNK association, found in nonstressed cells, limited the phosphorylation of Jun under nonstressed growth conditions. Treatment with H_2O_2 or UV light resulted in oligomerization of GST and the dissociation of the GST–JNK complex. JNK activity was found to increase in the presence of specific GST inhibitors. Overexpression of GST resulted in decreased JNK phosphorylation, concomitantly

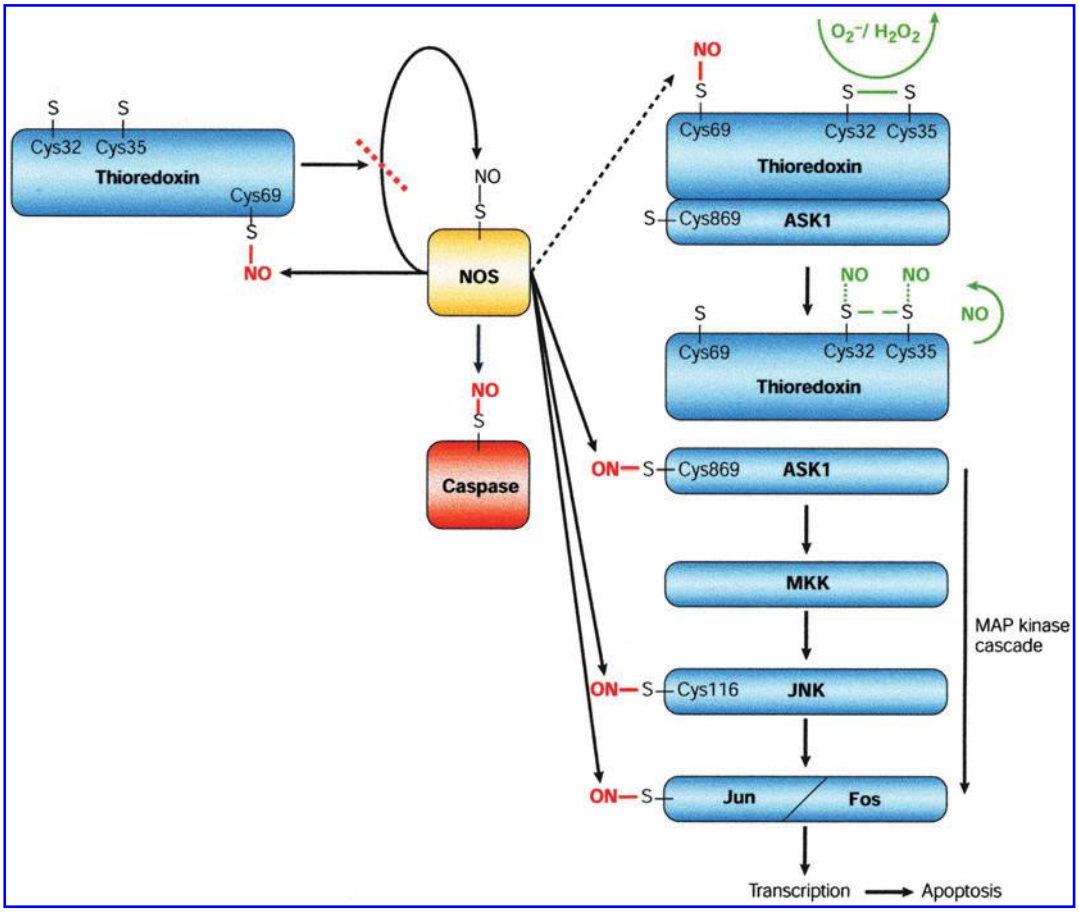

FIG. 3. Regulation of apoptosis through TRX-ASK1 by S-nitrosylation. TRX binds to ASK1, inactivating its apoptotic effect (see text for details). TRX can de-nitrosylate and thereby activate auto-S-nitrosylated NOS. In turn, NOS maintains the activity of TRX by S-nitrosylation of a cysteine residue in thioredoxin. In addition, S-nitrosylation inhibits the kinase activity of both ASK1 and JNK, and inhibits transcriptional activation by Jun. NOS activity can therefore propagate an antiapoptotic influence through S-nitrosylation of several elements in transduction pathways that are initiated by ASK1 and, in addition, exerts an inhibitory influence on other pro-apoptotic proteins, including caspases. TRX also exerts an antiapoptotic influence that is independent of ASK1 by scavenging reactive oxygen species (ROS; not shown). An oxidative stress, which is represented by ROS-induced modification of active-site Cys residues that inactivates TRX, exerts a pro-apoptotic effect. This is promoted, in part, by the release and activation of ASK1 and the subsequent transduction through the MAPK cascade that involves the sequential phosphorylation of MAPK kinase (MKK), JNK, and AP-1 (Jun-Fos). NO is colored red. Reprinted with permission from Macmillan Publishers Ltd. (122), Copyright (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

with decreased JNK activity, increased ubiquitination of c-Jun, and decreased c-Jun-mediated transcription. Free radical scavengers maintain the GST–JNK association and consequently JNK inhibition by GST (7).

b. Redox regulation of AP-1–DNA interactions. The interactions of AP-1 with its DNA-binding sequence are subject to redox regulation. DNA binding of the Fos-Jun heterodimer was found to be modulated by reduction–oxidation of the single conserved cysteine residue in the DNA-binding domains of the two proteins. The DNA-binding activity of Fos and Jun can be enhanced by mutating the Cys-154 residue in Fos and Cys-272 in Jun, both residing in the respective DNA-binding sites, into serine residues, or by treatment with high concentrations of reducing agents (5–10 mM DTT). These cysteine residues are located in a highly conserved sequence (Lys-Cys-Arg), generating a local environment that is predicted to significantly enhance the reactivity of cysteine residues, rendering these residues potentially highly susceptible to oxidation. It has been found that in v-Jun, the conserved cysteine residue is replaced with serine, suggesting that the transforming potential of v-Jun may result, in part, from the deregulation of DNA binding, due to the loss of the regulatory cysteine residue (2). *In vitro* DNA-binding activity of Jun and Fos has been found to be sensitive to oxidation (using 60–70 μM diazene dicarboxylic acid *bis*[*N*,*N*-dimethylamide] (diamide)) (2, 256). Moreover, mutating the critical cysteine residue in the *Fos* gene resulted in an increase in the affinity of *Fos* binding to DNA, rendering it redox independent, whereas the ability of the protein to induce transformation of cells was enhanced. These results suggested that redox regulation may limit the total level of functional Fos–Jun complexes *in vivo* and that escape from this control enhances transforming activity (256).

c. Regulation of the AP-1–DNA interactions through the action of APE1/Ref-1. Another level of redox regulation is presented through the action of APE1/Ref-1, a multifunctional protein possessing both DNA repair and transcriptional regulatory activities and has a pleiotropic role in controlling cellular response to oxidative stress. APE1 is playing a central role in the DNA base excision repair pathway of all DNA lesions. APE1/Ref-1 is involved in modulating genes' expression, regulated by major transcription factors, including AP-1, Egr-1, NF-κB, p53, HIF, and others, by reducing their critical cysteine residues [recently reviewed in Ref. (328)]. Ref-1 stimulates AP-1 DNA-binding activity through the reduction of conserved Cys residues in Fos and Jun, without altering their DNA-binding specificity (373). APE1/Ref-1 targets for reduction, in the c-Jun/c-Fos AP-1 heterodimer, are the Cys-272 of c-Jun and Cys-154 of c-Fos, conserved at their bZIP-type DNA-binding domains. This effect is accomplished through a redox cycle, in which TRX restores the reduced form of APE1/Ref-1. Co-overexpression of TRX and Ref-1 in COS-7 cells potentiated AP-1 activity only after TRX was transported into the nucleus by phorbol 12-myristate 13 acetate treatment, indicating the role of Ref-1–TRX interaction in the activation of AP-1 (124, 328, 347). Studies of the interactions between TRX and Ref-1, using both an *in vitro* cross-linking analysis and a mammalian two-hybrid system, with various mutants of TRX, have demonstrated the physical association of TRX and Ref-1 (124). *In vitro* analyses have revealed that Cys-65 is the redox-active site of APE1/Ref-1 (102, 359). In the 3D model structure of the protein, Cys-65 appears to be buried (109, 328). It was therefore assumed that the redox regulation requires conformational change of APE1/Ref-1 to allow interaction with the redox-sensitive transcription factor. A recent report (12) revealed that in the presence of GSH, APE1/Ref-1 enhanced DNA-binding activity of the c-Jun/c-Fos heterodimer independently of the cysteine residues of APE1/Ref-1, suggesting that the DNA-binding activity of AP-1 is promoted not only by the redox activity of APE1/Ref-1 (373). These studies demonstrated that both c-Jun and the c-Fos were only weakly reduced by GSH alone and that their reduction was promoted by both wt APE1/Ref-1 and a Cys-mutated APE1/Ref-1, at concentrations as low as 0.5 μM, in which APE1/Ref-1 alone could not reduce c-Jun and c-Fos. As is also discussed for APE1/Ref-1 interactions with NF-κB, in the following section, these results revealed a new activity of APE1/Ref-1, termed "redox chaperone" activity (12). The function of APE1/Ref-1 has been documented in the regulation of other transcription factors described below. Its further discussion in the following sections will be limited to features that are specific for the discussed transcription factor.

4. Involvement of RNS in AP-1 regulation. Nitric oxide (NO)-mediated regulation of AP-1 has been reported both *in vitro* and in a variety of cell lines *in vivo* [for review see Refs. (45, 64, 94, 122, 222, 223, 236, 263, 310)]. It has been found that the single-conserved-cysteine residues, Cys-272 and Cys-154, in AP-1 c-Jun and c-Fos, respectively, are subjects to NO-mediated regulation of AP-1 transcriptional activity. Inhibition of AP-1–DNA binding was observed after NO treatment (at concentrations $>140\,\mu$M), whereas DNA binding by a cysteine to serine mutant heterodimer lost its sensitivity to treatment with NO, implying that inhibition of AP-1–DNA interactions was mediated by nitrosylation of the redox-sensitive, conserved cysteine residues (251). Inhibition of c-Jun DNA-binding and AP-1 transcriptional activity has been found to be the result of NO-dependent glutathionylation [reviewed in Refs. (73, 100, 169)] at the cysteine residues of c-Jun, leading to the loss of c-Jun DNA-binding activity. Formation of mixed disulfide was found to display a virtually identical redox dependency and to map to the same cysteine residue located at the DNA-binding site of c-Jun, indicating that S-glutathionylation of this cysteine residue may account for the GSH/GSSG-mediated redox regulation of c-Jun DNA binding, and suggests the regulation of c-Jun DNA binding by reversible S-glutathionylation (169, 170).

In neurons and neural-derived tumor cells, NO was found to activate c-Fos-regulated transcription. *In vivo*, both exogenously provided NO donor (20 mM of 1,3-morpholino-sydnonimine [SIN]) (371) and endogenous NO, produced in response to various stimuli, induced c-Fos expression, whereas the inhibition of NO synthesis decreased c-Fos expression in neurons. Data obtained in various experimental systems suggested a special relationship between levels of NO production and regulation of c-Fos expression in neurons, mediating different types of painful sensations, as well as other stimuli [recently reviewed in Ref. (64)].

Similarly to the effects of ROS, various forms of RNS readily activate JNK (45). However, evidence suggest that the effect of RNS on JNK depends on various factors, including the type of RNS species, its level, the cell type, and the presence ROS [reviewed in Ref. (310)]. Early reports described JNK activation in response to exposure to exogenous NO donors, as well as enhanced (25–38-fold) cytotoxic levels of endogenously produced NO from inducible nitric oxide synthase (iNOS) (61). It was also reported that shear stress induced the formation of both NO and O_2^- in the cell, which react to yield peroxynitrite, which was suggested to promote the activation of JNK. Scavengers of NO and O_2^- prevented the shear-stress-dependent increase in tyrosine nitration (240) and activation of JNK (105).

It has been reported that S-nitrosylation by S-nitrosoglutathione (GSNO, $\sim 100\,\mu$M) suppresses JNK2 kinase activity, whereas thiol group (10 mM DTT) reductants can reverse this suppression (315). Studies on the NO negative regulation of JNK by S-nitrosylation have indicated that IFN-γ may function as a natural inhibitory signal for the JNK/SAPK pathway in macrophages and other iNOS-inducible cells, and that endogenous NO mediates the IFN-γ-induced suppression of JNK/SAPK through thiol-redox regulation (268). It was further demonstrated that NO disrupts the physical interaction between JNK and its substrate c-Jun *in vitro* (in the presence of 100 μM *S*-nitroso-*N*-acetylpenicillamine [SNAP]). The inhibition of the binding between JNK and c-Jun may be important in the mechanism underlying the negative regulation of the JNK signaling pathway by NO (270).

Overall, ROS and RNS affect the binding of AP-1 to its specific binding sites in the DNA and thereby regulate transactivation via both direct and indirect routes. First, they affect expression of the genes encoding AP-1 subunits. Second, they regulate AP-1 interactions with promoters and enhancers of their target genes directly, through the oxidation or reduction of cysteine thiols at the AP-1 DNA-binding domain in the protein (Cys-272 in Jun and Cys-154 in Fos). In this context, the function of APE1/Ref-1, in maintaining the

reduced form of critical cysteine residues in the protein-binding domain, is an important regulatory component. Third, they affect AP-1 activation indirectly via two major pathways: (a) through the upstream pathways of three groups of MAPKs, ERK, JNK, and p38, which are involved in the regulation of AP-1 and are activated in response to the intracellular redox state and oxidative stress; (b) through their effects on chromatin remodeling, enhancing, or suppressing the interaction of AP-1 with target genes' promoters, by the redox regulation of HAT and HDAC (see the following section for further discussion).

B. Nuclear factor Kappa B

1. Structure and function of NF-κB. NF-κB is an evolutionary-conserved inducible transcription factor that plays a key role in the cellular responses to environmental changes. It functions in many biological processes, but plays a pivotal role in the immune system, regulating expression of cytokines, growth factors, T- and B-cell receptors (TCRs, BCRs), and many other proteins that function in the immune response. NF-κB also regulates expression of genes in many other biological pathways, affecting central developmental and physiological processes. Its action influences the physiology of normal cells and during disease [for review see Refs. (104, 120, 274)]. In mammalian cells, the NF-κB family consists of five related redox-sensitive transcription factors, which include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, c-Rel, and their various combinations of homodimers and heterodimers. Members of this family contain the N-terminal Rel homology domain (RHD), a ~300-amino-acid protein domain that mediates DNA binding, dimerization, nuclear localization, and interactions with the family's inhibitory proteins, known as IκBs (120, 250, 274). They regulate inducible gene expression by binding to unique DNA sequences in promoter and enhancer sites. NF-κB functions in response to a variety of stimuli, regulating genes that are involved in controlling both program cell death (apoptosis) and cell proliferation, cell adhesion, innate and adaptive immune responses, inflammation, tissue remodeling, and cellular responses to stress (104, 120, 274). NF-κB dimers are generally found in a complex with IκB in the cytoplasm of resting cells and they are activated after receptor stimulation, which leads to their nuclear translocation.

NF-κB has long been known as a redox-sensitive transcription factor (115, 144, 341). However, as discussed below, the role of redox in NF-κB regulation has become controversial. Originally, this controversy has probably stemmed from the apparently different nature of redox regulation of NF-κB in the cytoplasm and the nucleus. Whereas in the cytoplasm ROS has been described in multiple reports to enhance signal transduction pathways, leading to NF-κB activation and translocation into the nucleus, it was shown to significantly inhibit the capacity of NF-κB to bind the DNA in the nucleus, a capacity that is consequently restored through the action of reducing enzymes. The apparently paradoxical role of ROS in the regulation of NF-κB has been challenged in a series of reports, which have documented a lack of consistency, in the responses of different cell types to redox, and provided alternative explanations for the previously described effect of antioxidants on NF-κB activation. These studies raised questions regarding the universal applicability of the role of redox in the regulation of NF-κB activation [reviewed in Refs. (144, 267)].

2. Activation of NF-κB in the cytoplasm. In most cell types, NF-κB complexes are retained inactive in the cell cytosol, while bound to their IκBs inhibitors, in a complex in which their nuclear localization signal (NLS), which is located at the RHD, is masked (120, 274). NF-κB is activated through several different cellular pathways induced in response to distinct stimuli (Fig. 4). A signaling pathway, often named the canonical or classical pathway, is the IκB-dependent pathway, which is induced in response to various inflammatory stimuli, including TNFα, interleukin-1 (IL-1), TCR, and bacterial lipopolysaccharide (LPS) (120, 274). It is dependent on the activation of IκB kinase (IKK), an enzyme that consists of two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit IKKγ, named the NF-κB essential modifier (NEMO). IKK (mainly IKKβ) phosphorylates IκBα at its Ser-32 and Ser-36 residues and IκBβ at Ser-19 and Ser-23, leading to ubiquitination and degradation by the 26S proteasome (120). The free NF-κB is then translocated into the nucleus and interacts with promoter and enhancer regions of target genes.

Although the canonical NF-κB pathway is the main mechanism, by which NF-κB activity is regulated, noncanonical (alternative) signaling pathways of NF-κB activation have been observed (Fig. 4). Several stimuli, such as stimulation by CD40, lymphotoxin-β-receptors, B cell activating factor, LPS, and Epstein-Bar virus (EBV), induce a signaling pathway that is based on the processing of the p100 protein, which is a precursor to the p52 subunit of NF-κB. NF-κB-inducing kinase (NIK) (382) and IKKα are the acting kinases, and mainly RelB/p52 NF-κB heterodimers are generated and may regulate a distinct subset of NF-κB target genes (104, 120, 250, 274). A distinct NF-κB activation pathway, induced by UV light and some specific chemotherapeutic agents, is based on the mitogen-activated protein (MAP) kinase p38 and casein kinase II (CK II), which phosphorylate IκBα, leading to its signal-induced IκBα degradation. Another type of unique NF-κB activation pathway is induced by genotoxic stress (41, 138), as well as other stress conditions, including oxidative stress (372). Here, upon cellular stress, the IKK subunit NEMO is sumoylated in the nucleus, by covalent attachment of the small ubiquitin-like modifier (SUMO), and then ubiquitinated, in an ataxia telangiectasia mutated (ATM)-dependent process. NEMO with ATM are relocated to the cytoplasm, where NEMO activates the IKK complex (372).

3. The role of redox in the activation of NF-κB. The potential role of redox in the activation of NF-κB has been extensively studied (Fig. 5). An important level of ROS-dependent regulation of NF-κB activation is provided by a series of redox-sensitive protein kinases (115, 267) that promote the dissociation of the IκB–NF-κB complex by phosphorylation of either IκB or NF-κB. These include IKKα and IKKβ, the redox-sensitive MAPK/ERK kinase-1 (MEKK-1), and AKT, which is activated by phosphatidylinositiol-3-kinase (PI-3-K), depending on the cell type (144, 267, 341). A series of reports provided evidence for the redox regulation of NF-κB activation. These studies have shown that ionizing radiation or H_2O_2 activates NF-κB (137). H_2O_2 (at the levels of 100 μM) enhanced the activation of NF-κB by known stimuli, such as TNFα or phorbol myristate acetate (PMA). It was also observed

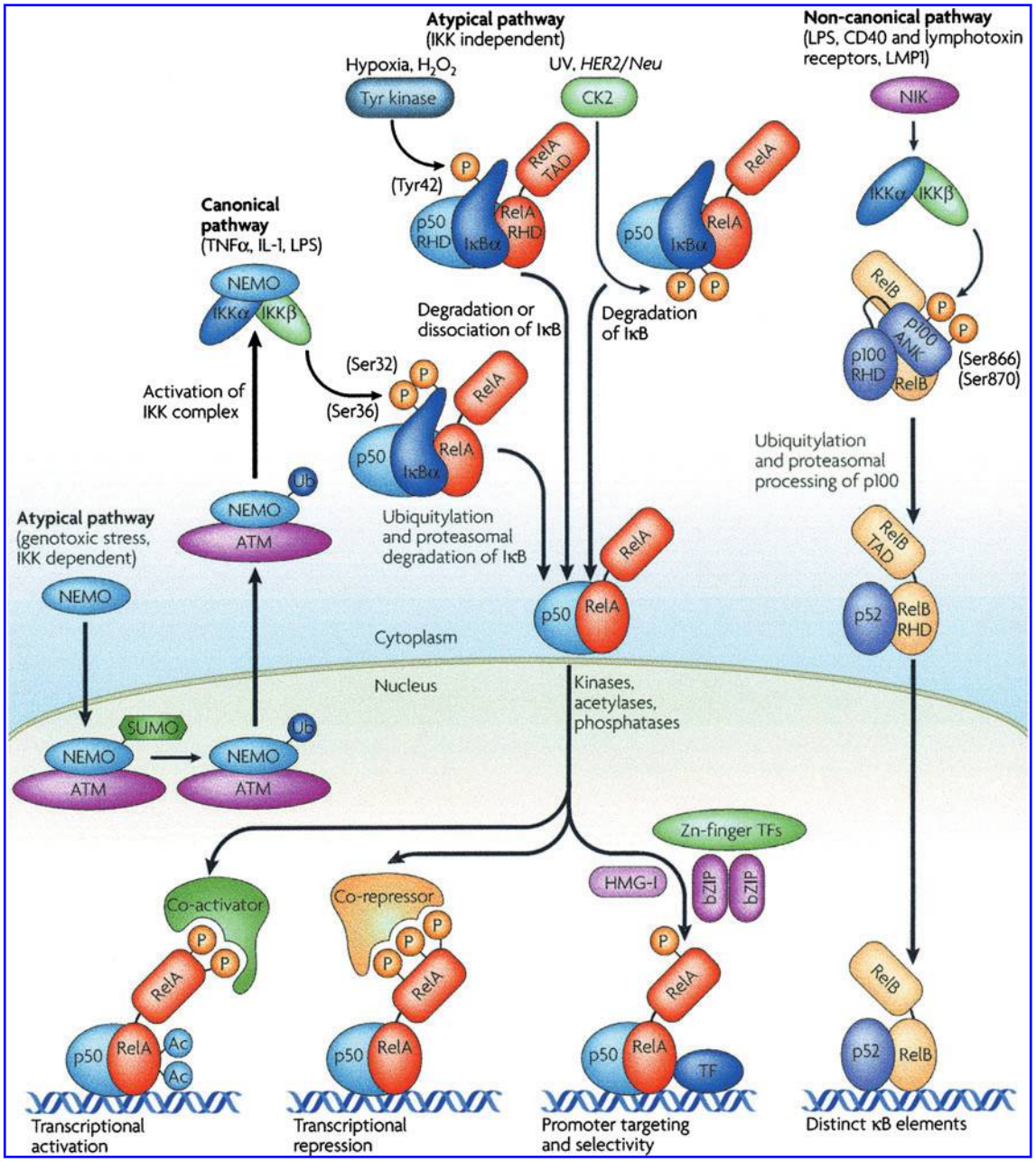

FIG. 4. Pathways leading to the activation of NF-kB. The canonical pathway is induced by TNFα, IL-1, and other stimuli, and is dependent on activation of IKKβ. This activation results in the phosphorylation of IκBα, leading to its Ub and subsequent degradation by the 26S proteasome. Release of the NF-κB complex allows it to relocate to the nucleus. IKK-dependent activation of NF-κB can occur following genotoxic stress. NEMO localizes to the nucleus, where it is sumoylated and then ubiquitylated, in a process that is dependent on the ATM checkpoint kinase. NEMO relocates back to the cytoplasm together with ATM, where activation of IKKβ occurs. IKK-independent atypical pathways of NF-κB activation include CK II and tyrosine-kinase-dependent pathways. The noncanonical pathway results in the activation of IKKα by the NIK, followed by phosphorylation of the p100 NF-κB subunit by IKKα. This results in proteasome-dependent processing of p100–p52, which can lead to the activation of p52–RelB heterodimers that target distinct κB elements. ATM, ataxia telangiectasia mutated; IL-1, interleukin-1; NEMO, NF-κB essential modifier; NIK, NF-κB-inducing kinase; P, phosphorylation; TNFα, tumor necrosis factor-α; Ub, ubiquitylation. Reprinted with permission from Macmillan Publishers Ltd. (274), Copyright (2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

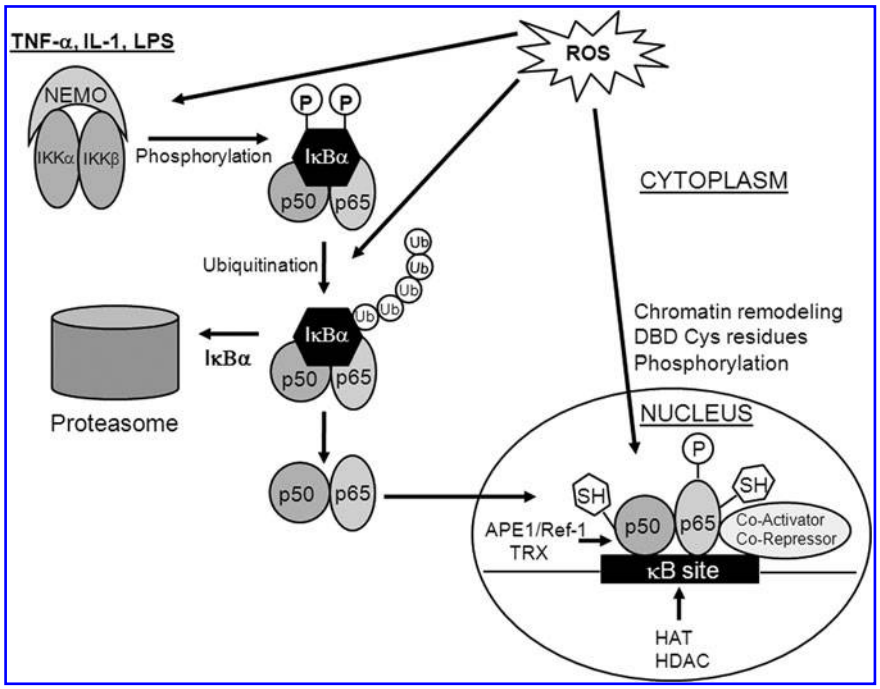

FIG. 5. Redox regulation of NF-κB. In nonstimulated cells, NF-κB is found in the cytoplasm in an inactive form, in association with its inhibitor IκB. In the canonical pathway, which is induced in response to various inflammatory stimuli, including TNFα, IL-1, and LPS, IKK that consists of the catalytic subunits, IKKα, and IKKβ and the regulatory subunit NEMO phosphorylates IκBα, leading to its ubiquitination and degradation by the 26S proteasome. ROS activates IKK directly, through redox modification of IKK, or indirectly, through activation of AKT and/or MEKK1. Active free NF-κB translocates to the nucleus. In the nucleus, oxidation of cysteine residues in the DNA-binding domain (Cys-62 in p50 and Cys-38 in p65) inhibits the binding of NF-κB to its DNA-binding sites. This inhibition is released through the action of APE1/Ref-1 and TRX. ROS can also inactivate the HDAC activity, shifting the balance of HAT/HDAC toward histone acetylation and thereby chromatin uncoiling, increasing the accessibility for transactivation by NF-κB. APE1/Ref-1, AP endonuclease 1/redox factor 1; DBD, DNA-binding domain; HDAC, histone deacetylases; IκB, inhibitor kappa B; NF-κB, nuclear factor kappa B; P, phosphorylation.

that a decrease in the intracellular pool of GSH, an important protective antioxidant, followed by a subsequent increase in the levels of GSSG, mediates IκBα phosphorylation and subsequent activation of NF-κB (283). Another intracellular antioxidant, *N*-acetyl-L-cysteine (NAC, 30 mM), which counteracts the effects of ROS in the cell repressed TNFα-induced NF-κB activation (254). Studies of NAC, as well as other thiol compounds and of metal chelators (11, 300, 317), showed that they also blocked the activation of NF-κB by cycloheximide, double-stranded RNA, calcium ionophore, TNF-α, phorbol ester, interleukin-1, and LPS, suggesting that diverse agents that activate NF-κB by distinct intracellular pathways may act through a common mechanism that involves the synthesis of ROS. Activation of NF-κB by TNFα or IL-1β led to a burst of ROS production, through the activation of NADPH oxidases in a process mediated by the Toll-like receptor 4 (TLR4) that contributed to NF-κB activation (269). Under TNF or IL-1 induction, intracellular ROS levels rise and ROS production upregulates the JNK-mediated NF-κB activation. It was also reported that Rac1 (Ras-related C3 botulinum toxin substrate 1), a GTP-binding protein, regulates NF-κB activity by activating JNK, through the phosphorylation of JNK by the MAP kinase kinase (MEKK1). Rac proteins also regulate ROS production by NADP oxidase. Rac-induced ROS production is also required for NF-κB activation upon IL-1 or LPS stimulation [reviewed in Ref. (144)].

Overall, the data accumulated supported the involvement of ROS in the activation of NF-κB. Nevertheless, as mentioned above, the role of redox in NF-κB regulation remained under debate [reviewed in Refs. (115, 267)] due to a series of studies whose results are contradictory to those described above. Several studies have shown that H_2O_2-induced NF-κB activation is highly cell-type dependent (189), implying that NF-κB activation is apparently not a universal response to ROS. Hayakawa *et al.* (119) examined the redox-dependent inhibition of NF-κB activation by antioxidant compounds, such as NAC (30 mM) and pyrrolidine dithiocarbamate (PDTC, 200 μM), demonstrating that both NAC and PDTC inhibit NF-κB activation independently of their antioxidative function. While NAC was found to block TNF-stimulated NF-κB activation by inhibiting the binding of TNF to its receptors, PDTC was found to inhibit ubiquitin ligase activity on phosphorylated IκBα in a cell-free system (115, 189, 267). It has also been suggested that treatment with H_2O_2 (at the levels of 100 and 500 μM) in airway epithelial cells caused a general inhibition of proteasomal activity (139). The role of ROS as second messengers, leading to NF-κB activation in response to extracellular stimuli, was also reexamined, in cell lines in which the endogenous ROS level could be controlled by blocking or activating the Rac/NADPH oxidase system. On the basis of these studies it was suggested that endogenously produced ROS do not mediate NF-κB activation (119).

4. Transcriptional activation of NF-κB

a. Binding of NF-κB to κB DNA targets. Upon activation of NF-κB and proteasome-mediated degradation of IκB, the free NF-κB is translocated to the nucleus and activates transcription by binding to sequence-specific target DNA, known as κB DNA, at gene promoters and enhancers. κBs are 10 bp elements of the consensus sequence 5′-GGGRNWYYCC-3′ (where R = purine, N = any base, W = A/T, and Y = pyrimidine), of which hundreds of variations exist in gene promoters. They are divided into two broad classes. Class I κB DNA binds optimally to p50 and p52 NF-κB subunits, whereas class

II κB DNA is recognized by c-Rel and p65. Different NF-κB dimers show significant overlap in binding to κB DNA variants *in vitro*. Availability of X-ray structures of several NF-κB homodimers and heterodimers, bound to different κB DNA sequences (52, 128, 241), enabled a better understanding of the rules governing the preferential DNA target recognition by different NF-κB dimers and the specificity in NF-κB transactivation activity. Overall, these observations suggest that NF-κB dimers recognize specific structural features of κB DNA to generate sequence-specific complexes. Small changes in promoter-specific κB DNA, of even a single nucleotide, can alter the gene expression profile (51, 186, 334). It has been suggested that the sequence of the κB site plays an important role in determining which coactivators will form productive interactions with the bound NF-κB dimer, rather than determining the ability of a particular dimer to bind the site effectively (186). The impact of a single-nucleotide change within the κB site on the binding affinity in the interaction of NF-κB with its binding sites in the DNA has been recently demonstrated in the work of Udalova *et al.* (346). These investigators have reported on the application of a method that predicts the effects of single-nucleotide polymorphisms within regulatory sequences on protein–DNA interactions to the analysis of the interactions of NF-κB with its κB-binding sites. Prediction accuracy was also verified experimentally by electrophoretic mobility-shift assay (EMSA). Their analyses of the interactions of variants in the κB-binding motif with recombinant p50/p50 homodimer and p50/p65 heterodimer revealed that, depending on context, a single-nucleotide change between two κB sequences can cause changes in binding affinities, varying from small changes up to significant ones (346). The crystal structure of the p50/p65 heterodimer, which is the most abundant of the Rel/NF-κB dimers, bound to the immunoglobulin (Ig)-κB (52), provided structural information regarding the binding modes of κB DNA targets. It revealed that each subunit consists of two Ig-like domains connected by a 10-amino-acid flexible linker. Conformational adjustments in both subunits are necessary for base-specific binding to the κB targets. The subunits linker regions can adjust the N-terminal domain so that the DNA-contacting residues are aligned properly and each sequence is bound in a way that preserves the protein–DNA interface chemistry (52). In accord with these observations are reports demonstrating significant differences in the bending of p50/p65 heterodimer, bound to distinct κB targets, containing small sequence differences (90). Analyses of the X-ray structures of various NF-κB dimers, bound to different κB DNA sites (128), reveal significantly distinct conformations. The same dimer, bound to κB DNA sites with only small sequence variations, displayed strikingly different conformations. On the basis of these observations it was suggested (128) that the simple occupation of a DNA site by NF-κB *per se* is not sufficient to drive transcription (51, 334) and that the conformation and flexibility of κB DNA sequences play a critical role in the recognition of NF-κB (128).

b. Effect of posttranslational modifications on NF-κB transactivation. NF-κB subunits are subject to modifications and interactions with coactivators, corepressors, and selective cointeraction with heterologous transcription factors to specific promoters and enhancers, defining their specificity in regulating transcription of target genes. The integration, or crosstalk, of NF-κB pathway with other major signaling pathways in the cell, including the JNK-signaling pathway, p53 tumor suppressor, and with nuclear receptors, which have important impact on its activity, has been the subject of several recent reviews (104, 120, 274, 275).

NF-κB is covalently modified by direct phosphorylation and acetylation, altering the ability of NF-κB dimers to bind DNA and initiate the recruitment of transcription coactivators (55, 104, 120). RelA phosphorylation has been studied in detail [reviewed in Ref. (55)]. A key phosphorylation event that regulates both the DNA-binding and oligomerization properties of NF-κB is the phosphorylation of Ser-276 residue in the RHD of RelA, in the cytoplasm, by the catalytic subunit of protein kinase A (PKA), in response to LPS-induced stimulus (101). PKCζ phosphorylates this site in NF-κB in the nucleus, in response to TNFα. RelA is phosphorylated by multiple distinct kinases that specifically affect its transactivation potential, including the PKA and PKCζ (243) mentioned above, mitogen and stress-activated kinase-1 (MSK-1) (303), CK II (138), RSK-1 (13), GSK-3β (23), and AKT/(PI-3-K) (48). NF-κB phosphorylation by several of these kinases is significantly affected, through their oxidation, glutathionylation, and S-nitrosylation (55, 267). The redox regulation of protein kinases that are involved in the control of NF-κB activation has been discussed extensively elsewhere (267). It includes protein kinases involved in NF-κB activation in the cytoplasm, as well as those affecting its transactivation activity in the nucleus. For example, covalent modifications of the Cys-179 residue in IKKβ by cyclopentenone prostaglandins inhibits its kinase activity and subsequent NF-κB DNA binding (IC_{50} concentration of PGA1 toward NF-kB was 12 mM in Jurkat cells) in response to TNFα, IL-1β, and TPA (291). Oxidation of Cys-179 of IKKβ in HeLa and HEK293 cells results in the inhibition of NF-κB. Arsenite potently inhibits NF-kB and IKK activation by binding to Cys-179 in IKK catalytic subunits (IC_{50} of 8.7 mM toward NF-kB DNA-binding activity and 9.1 mM toward IKK) (154). S-nitrosylation inhibited IKK activity and NF-κB transactivation in mouse lung epithelial cells and Jurkat T cells. Both SNAP and S-nitrosoglutathione (GSNO) caused a dose-dependent decrease (at the levels of 0.1–1.0 mM) in IKK enzymatic activity *in vitro* (289). An IKKβ kinase, MEKK-1, is redox sensitive and can be activated by TNFα and IL-1β, activators of the canonical NF-κB pathway. Glutathionylation of the enzyme [using 250 μM menadione (2-methyl-1,4-naphthoquinone)] at its Cys-1238 residue, located in the enzyme ATP-binding domain, leads to its inhibition (67). AKT has been found to increase NF-kB transactivation through activation of IKKβ (211). AKT contains a redox-sensitive kinase domain. In cardiac cells exposed to H_2O_2, formation of a disulfide bond between Cys-297 and Cys-311 led to inactivation of AKT and apoptosis. AKT activation in response to ROS (0.4–2.0 mM H_2O_2) depends on platelet-derived growth factor (PDGF) receptor or epidermal growth factor (EGF) receptor activation, and peroxynitrite (at the level of 500 μM) also activates of AKT in a dose- and time-dependent manner (172, 363). AKT activity is also affected by its upstream inhibitor phosphatase and tensin homolog protein, which is a redox-sensitive phosphatase [reviewed in Ref. (242)]. Regulation of NF-κB activity is also affected through the action of nuclear protein kinases that affect its transactivation activity. Data accumulated suggest a major role for PKCζ in the redox regulation of NF-κB activation. The PKC family members are targets of

cysteine oxidation, S-glutathionylation, and tyrosine nitration, which inhibit their activity (60, 173, 364). PKCζ activates IKKβ (182) and also phosphorylates RelA at the Ser-311 residue, which is essential for association of NF-κB with CBP (84). PKA was found to phosphorylate the RelA Ser-276, which is located near the NLS. Oxidation of two cysteine residues in PKAc catalytic subunit, Cys-199 and Cys-343 by diamide, generates an intramolecular disulfide bond and inhibits PKA's kinase activity (at the range of ~1.0–4.0 mM) (129). S-glutathionylation of Cys-199 also resulted in inhibition of its catalytic activity (129). RelA Ser-276, Ser-528, and Ser-536 residues are phosphorylated by MSK-1, RSK-1, and CK II, respectively, affecting NF-kB transcriptional activity, in response to stimuli such as TNFα, IL-1, or p53. MSK-1 and RSK activity have been found to be activated as a result of exposure to H_2O_2. CK II was also linked to the redox regulation of NF-κB [reviewed in Ref. (267)].

NF-κB subunits are also acetylated on multiple lysine residues that regulate distinct functions of NF-κB, including transcriptional activation, DNA binding, and subcellular localization (54). Three main acetylation sites, Lys-218, Lys-221, and Lys-310, have been identified in RelA in response to TNFα or PMA stimulation. Acetylation at Lys-221 enhances DNA binding by RelA and impairs its assembly with IκBα, whereas acetylation of Lys-310 is required for full transcriptional activity of RelA, but does not affect DNA binding or IκBα assembly. Acetylation of Lys-310 is significantly enhanced by prior phosphorylation of Ser-276 and Ser-326 (56, 390). RelA acetylation is mediated by the p300/CBP-associated factor (PCAF) acetyltransferase (54, 159, 379). Acetylated RelA displays a higher affinity for DNA and a decreased binding affinity for IκBα. Acetylation of p50 on Lys-431, Lys-440, and Lys-441 enhances the DNA-binding and transcription activity by the NF-κB heterodimer (55). Acetylation of RelA/p65 is a dynamic process where the acetylation status of specific lysine residues affects both the DNA-binding ability and transcriptional activity of NF-κB (54, 159). Class I histone deacetylases HDAC1-3 deacetylate RelA/p65 NF-κB, resulting in loss of transcriptional activation potential, increased association with IκBα, or enhanced nuclear export of NF-κB (15, 54, 390). SIRT1, the mammalian ortholog of the yeast silencing information regulator 2 (SIR2), a nicotineamid adenine dinucleotide-dependent HDAC, interacts with the RelA/p65 subunit of NF-κB. It regulates NF-κB-dependent transcription and cell survival in response to TNF-α by RelA/p65 deacetylation at Lys-310 (53, 378).

5. Redox regulation of NF-κB–DNA interactions and transactivation. Whereas ROS can mediate the upregulation of NF-κB activation in the cytoplasm, they decrease NF-κB DNA-binding activity in the nucleus. Redox affects NF-κB–DNA interactions at several levels of regulation (Fig. 5). Oxidation of the p50 subunit Cys-62 residue inhibits DNA binding. This cysteine residue, located in the conserved RHD of NF-κB, is oxidized in the cytoplasm and after stimulation it is converted into the reduced form in the nucleus to gain DNA-binding activity. Thus, within the DNA-binding domain, the Cys-62 residue of p50 subunit is critical for ROS-regulated DNA binding. Inactivation of NF-κB DNA-binding activity occurs also as a result of glutathionylation (using the GSH/2GSSG redox pair at different ratios, at the range of 100–0.1 and total of at 3 mM GSH equivalents) (277). S-nitrosylation (by 1–5 mM sodium nitroprusside [SNP] or 0.01–1 mM SNAP) was also reported (222, 225), providing another control mechanism for modulating expression of NF-κB-responsive genes [reviewed in Ref. (144)]. It has been found that the promoter of the murine gene encoding iNOS contains an NF-κB site, which is necessary for LPS inducibility of the iNOS promoter. Hence, activation of NF-κB induces a large amount of NO production by upregulating the expression of inducible NO synthase (374). NO production can serve as a negative regulator of NF-κB activation in the nucleus. While the redox-sensitive Cys-62 residue in the p50 monomer was considered to be the target for NOS-2-mediated S-nitrosylation in the p50–p65 heterodimer, it has been recently shown that the p65 (RelA) is S-nitrosylated in cytokine-stimulated respiratory epithelium and macrophages on the conserved Cys-38 residue within the DNA-binding site at the RHD. S-nitrosylation of p65 is dependent upon NOS 2 activity, and the levels of nuclear S-nitrosothiol (SNO)–p65 are inversely correlated with NF-κB p50–p65 DNA binding and NF-κB-dependent transcription, suggesting a role for NOS 2 in regulating NF-κB-dependent transcription of response mediators (158). It was also reported that GSNO, an endogenous nitric oxide carrier, mediated nitrosylation of p65, inhibiting its binding at NF-κB consensus sequence and thereby its transcriptional regulatory activity (281). Earlier studies have shown that TRX reduces the oxidized p50 subunit and restores DNA-binding activity of NF-κB. TRX upregulates NF-κB activation in the nucleus, whereas downregulating NF-κB activation in the cytoplasm (144). Early studies have demonstrated that cotransfection of a plasmid expressing human TRX and an HIV LTR-driven reporter construct resulted in an NF-κB-dependent increase in expression of the reporter gene, indicating that thioredoxin can regulate the NF-κB DNA-binding activity *in vivo* (226).

A recent report links the regulatory effect of H_2O_2 to the binding affinities in the interactions of NF-κB with various κB sites (258). Observations in MCF-7 and HeLa cells have revealed that action of H_2O_2 in the activation of NF-κB is biphasic. Extracellular concentrations of H_2O_2 of up to 25 μM did not induce significant translocation of NF-κB to the nucleus, but stimulated the translocation induced by TNF-α, while higher H_2O_2 levels resulted in its inhibition. It has been observed that during inflammation, the *in vivo* concentration of 12.5 μM H_2O_2 upregulated expression of several pro-inflammatory NF-κB-dependent genes induced by TNF-α (257). It has also been found that H_2O_2 regulates inflammation via upregulation of some NF-κB-dependent genes, whereas expression of most other NF-κB-dependent genes remained unaltered (258). On the basis of these observations and the recent report demonstrating the strict sequence dependence of the binding affinity in NF-κB interaction with its binding site (346) as discussed above, Oliveira-Marques *et al.* (258) tested a hypothesis that by increasing NF-κB translocation into the cell nucleus, H_2O_2 shifts the equilibrium toward a higher κB site occupancy, and that differential gene expression is affected by the affinity of κB sites to NF-κB. Hence, NF-κB interactions with low-affinity κB sites should be enhanced by H_2O_2, whereas interactions with high-affinity κB sites should not be affected. This hypothesis was examined by transfection of cells with plasmids coupled to a reporter gene, which contains κB sequences with three different affinities in the interaction with NF-κB. Cells were exposed to TNF-α in the absence or

presence of a H_2O_2. On the basis of the results obtained, it was predicted that genes with high-affinity κB sites are insensitive to H_2O_2, whereas genes with lower-affinity κB sites are upregulated by H_2O_2 (258).

Another level of redox regulation is presented through the action of APE1/Ref-1 (see section II.A.3.c for further discussion of APE1/Ref-1 activity). It has been observed that the redox chaperone activity of APE1/Ref-1 is critical to NF-κB-mediated gene expression and is conducted through its physical association with the transcription factor (12). It has been demonstrated that the redox state of NF-κB is spatially regulated by its subcellular localization. While cysteines in the p65 subunit and most residues in the p50 subunit are reduced similarly in the cytoplasm and in the nucleus, Cys-62 of p50, which is essential for the DNA-binding activity of NF-κB, is oxidized in the cytoplasm and reduced in the nucleus. Maleimide labeling of the oxidized NF-κB p50 subunit clearly showed that Ref-1 is involved in Cys-62 reduction in the nucleus (252).

6. Redox effect on the targeted modulation of chromatin structure. An additional level of regulation of NF-κB transcriptional activity that was found to be affected by redox is represented by the targeted remodulation of chromatin structure. The condensation of eukaryotic DNA in chromatin suppresses gene expression by decreasing the accessibility of transcription factors, such as NF-κB, and of the transcription apparatus, to initiate the transcription of target genes. Acetylation of lysine residues on the N-terminal tails of the core histones results in a decrease in affinity of histone–DNA interactions in the nucleosome, recruitment of switch/sucrose nonfermentable (SWI/SNF) remodeling factors, and, subsequently, the uncoiling of the nucleosomal complex (229). This process, known as chromatin remodeling, allows increased accessibility of transcription factors and the replication machinery to the unwound complex and promote the initiation of gene transcription. Acetylated lysines are specifically recognized by bromodomains containing proteins, such as coactivators (153). Histone acetylation is reversible and the balance of chromatin acetylation/deacetylation is determined through the action of HAT, which catalyzes histone acetylation and that of HDAC, which catalyzes their deacetylation. The transcriptional coactivator CBP/p300 (see above), which contains an intrinsic HAT activity, is regulated through the p38 MAP kinase. Phosphorylation of CBP/300 results in its activation, and the NF-κB–coactivator complex formation results in increase in targeted chromatin acetylation, which leads to local unwinding of the chromatin structure and facilitates the access of transcriptional machinery to the promoter of NF-κB-regulated genes.

Redox regulation of NF-κB activity, at the level of chromatin remodeling, has been extensively discussed recently [for review see (6, 284, 286)]. Redox plays an important role in the regulation of the balance of histone acetylation/deacetylation, through its effect on the activities of HAT and HDACs. Consequently, redox affects chromatin remodeling and the specific activation of NF-κB-mediated gene expression (54, 253). The CBP/p300 coactivator, functioning as a HAT, is regulated by the p38 MAP kinase pathway that is activated by oxidative stress condition (284). Oxidative stress mediates posttranslational modifications, such as nitration and carbonylation of HDACs and SIRT1 (378). These modifications reduce HDACs activity and lead to increased ubiquitination and subsequent degradation, which consequently increases the ratio of histone acetylation/deacetylation, and thereby increases chromatin remodeling (131, 377). In addition, HDAC1 is associated with NF-κB p65 (15, 390). The oxidative stress-mediated phosphorylation of RelA/p65 can lead to its dissociation from HDAC1 and its association with CBP/p300 (377, 390), leading to histone acetylation and promote NF-κB-mediated gene expression. Regulation of gene expression at the level of the chromatin structure has been also documented in the case of other transcription factors. Its further discussion throughout this review is limited to features that are specific to the individual transcription factors.

Overall, the data described above demonstrate that redox signaling plays an essential role in the control of NFκB activity at several levels. This includes the regulation of its interactions with κB sites in target genes, both directly, by affecting the redox state of cysteine thiols at its DNA-binding domain, and indirectly, via its effect on chromatin structure. However, the data presented also reflect the fact that the role of redox in NF-κB regulation has been controversial. This controversy has focused especially on the process of NF-κB activation in the cytoplasm. In this stage, a series of redox-sensitive protein kinases, including IKKα and IKKβ, MEKK-1, and AKT, have been reported to promote the dissociation of the IκB–NF-κB complex by phosphorylation of either IκB or NF-κB, in a cell-type-dependent manner. The different responses of various cell types to ROS and questions regarding the inhibitory effects of antioxidants on NF-κB activation raised doubts concerning the universality of NF-κB activation in response to ROS. A major factor in this controversy has been the apparently paradoxical role of ROS in the overall regulation of NF-κB, where it may upregulate NF-κB activation in the cytoplasm, but inhibits NF-κB DNA-binding activity and thereby transactivation in the nucleus. Inhibition of NF-κB binding to its responsive element was found to occur through the oxidation of the Cys-62 residue in the p50 subunit and via its S-nitrosylation, as well as through the S-nitrosylation of Cys-38 in the p65 subunit, both within the DNA-binding domains of the corresponding subunits. Reduction of cysteine thiols by TRX and APE1/Ref-1 restores and upregulates NF-κB activity in the nucleus, providing an additional level of redox-mediated regulation. Finally, a recent report implicated ROS in yet another level of NF-κB regulation, linking ROS to the differential binding of NF-κB to its κB sites in the target genes.

C. Hypoxia inducible factor

1. Function of HIF. HIFs play a key role in the maintenance of oxygen homeostasis in metazoan organisms, by regulating the transcriptional response to changes in oxygen supply. They regulate expression of many genes, addressing the decrease in oxygen supply by facilitating both oxygen delivery and metabolic adaptation to oxygen deprivation [recently reviewed in Refs. (98, 146, 149, 197, 239, 341)]. These processes include the control of glucose uptake metabolism, vasculogenesis, angiogenesis, erythropoiesis, vasodilation, pH homoeostasis, autophagy, and decisions regarding cell survival, proliferation, or apoptosis. Thus, HIFs affect multiple signaling pathways that influence development, metabolism, inflammation, and cell and tissue physiology, and

multiple steps in tumorigenesis [reviewed in Refs. (98, 146, 239, 288)].

To optimize glucose and O_2 utilization in hypoxia and generate sufficient amounts of ATP, HIF-1α modulates key metabolic pathways. It directly controls multiple enzymes that are responsible for shifting the metabolism toward the less energy-efficient metabolism of anaerobic glycolysis in O_2-deprived cells. In addition, production of high amounts of ROS by mitochondrial respiration under the hypoxic conditions is attenuated due to HIF-1α-mediated downregulation of mitochondrial oxygen consumption. This is through the suppression of the TCA cycle and mitochondrial respiration by an HIF-mediated induction of pyruvate dehydrogenase kinase (PDK), which phosphorylates, and thereby inhibits, pyruvate dehydrogenase, preventing its entry into the TCA cycle [reviewed in Ref. (306)]. Moreover, HIF also affects mitochondrial respiration by upregulating, under hypoxic conditions, the expression of a more efficient cytochrome oxidase isoform, to replace the isoform functioning in normoxia, mediating its degradation by an HIF-1α-regulated LON protease (99) [recently reviewed in Ref. (18)]. The pivotal role played by HIF in the regulation of angiogenesis affects normal growth and development, wound healing, tissue and organ regeneration, and pathological processes, such as cancer. The role of HIF in several aspects of tumorigenesis, including angiogenesis, metabolism, proliferation, metastasis and differentiation, has been discussed extensively in recent years [for recent review see Refs. (98, 146, 239, 288, 365)].

2. HIF structure and regulation. Both HIF proteins stability and their transcriptional activity are oxygen dependent. They are significantly inducible under hypoxia and rendered almost inactive under normoxia. Nevertheless, HIF can also be activated by nonhypoxic stimuli, including hormones such as insulin, growth factors, coagulation factors, vasoactive peptides, and cytokines, under normoxia (280).

HIF is a heterodimer consisting of two basic helix-loop-helix (bHLH) protein subunits (Fig. 6) of the PER, ARNT, and SIM (PAS) family of transcription factors, an unstable HIF-α subunit, whose levels in the cell are strictly regulated in an oxygen-dependent manner, and a constitutively expressed HIF-β subunit (also known as the aryl hydrocarbon receptor nuclear translocator [ARNT1]), both containing transactivation domains (34). Metazoa contain three known HIF-α proteins, HIF-1α, HIF-2α, and HIF-3α, and three HIF-β proteins (Arnt1, Arnt2, and Arnt3). Both HIF-1α and HIF-2α can assemble with HIF-1β to form the functional HIF transcription factor complexes. HIF-3α has no known role as an active transcription factor and was suggested to play a role as a regulator of HIF transactivation. HIF-3α splice variants have been found to be inhibitory to HIF-dependent transcription. Such an alternative splicing event, yielding an inhibitory PAS domain protein (IPAS) that assembles with HIF-β, has been reported to be induced by hypoxic conditions and may function in the regulation of HIF transcriptional activity (212, 213). In hypoxia, such heterodimer binds to a pentanucleotide core sequence (RCGTG) in the hypoxia response elements (HREs) of HIF-regulated genes.

The HIF-α protein is constitutively expressed in the cell. However, under normoxic conditions, it has a rapid turnover as it is continuously synthesized and degraded. HIF degradation is mediated through the hydroxylation of either of two highly conserved proline residues, within the oxygen-dependent degradation (ODD) domain (Figs. 6 and 7), by HIF-α-specific prolyl hydroxylases, which are members of the prolyl hydroxylase domain (PHD) family [reviewed in Refs. (145, 297)]. Hydroxylation promotes HIF-α polyubiquitination through the generation of a binding site for the von Hippel–Lindau (pVHL) tumor suppressor protein, a recognition component for the ubiquitin ligase complex. As a result, under normoxic conditions HIF-α is tagged for proteasomal degradation. The PHD proteins that hydroxylate HIF-α belong to the Fe^{2+} and 2-oxoglutarate-dependent oxygenase superfamily, whose activity is dependent on oxygen. Hence, hypoxia suppresses the rate of HIF hydroxylation and consequently impedes the proteasomal degradation of HIF-α. Under low oxygen conditions, or in the absence of functional pVHL, HIF-α accumulates in the cytoplasm, where it is associated with the heat-shock protein 90 (Hsp90), which enhances its stability. Under such hypoxic conditions, HIF-α dimerizes with an HIF-β subunit and the heterodimer complex translocates to the nucleus, where it binds to the HRE in promoter or enhancer sequences and transcriptionally activates multiple HIF-regulated genes [reviewed in Ref. (197)].

Binding to DNA requires HIF dimerization and is mediated by the bHLH and PAS domains, allowing DNA binding by the adjacent basic sequences. HIF-1α and HIF-2α contain two

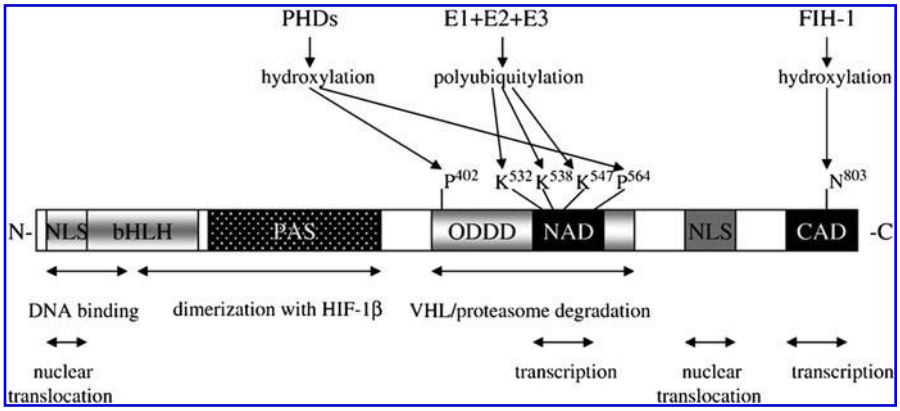

FIG. 6. HIF-1α structure. Schematic description of the domain structure of HIF-1α, the function of individual domains and the sites of posttranslational modifications that critically affect its function. bHLH, basic helix-loop-helix; CAD, C-terminal activation domain; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; FIH-1, factor inhibiting HIF-1; HIF, hypoxia-inducible transcription factor; NAD, N-terminal activation domain; NLS, nuclear localization signal; ODDD, superoxide (O_2)-dependent degradation domain; PAS, PER-ARNT-SIM; PHDs, prolyl hydroxylases. Reprinted with permission from Elsevier (149), Copyright (2008).

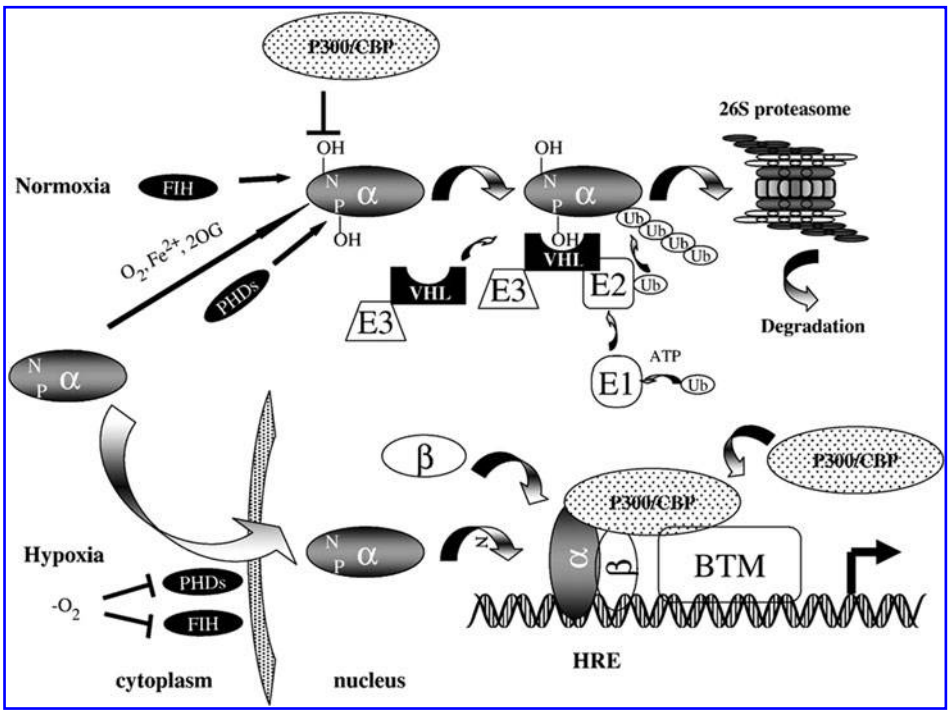

FIG. 7. The outline of regulation of stability and transcriptional activity of HIF-α. In the presence of O_2 and cofactors Fe^{2+} and 2OG, PHDs hydroxylate HIF-α, allowing its ubiquitination by the E3 ubiquitin ligase, and degradation in the 26S proteasome. FIH-1-mediated N-hydroxylation prevents recruitment of p300/CBP transcriptional coactivators. In the absence of O_2, PHDs and FIH-1 are inactivated and nonhydroxylated HIF-α translocates to the nucleus, dimerizes with HIF-β, recruits p300/CBP, and induces expression of its target genes via binding to the HRE. BTM, basic transcriptional machinery; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; FIH-1, factor inhibiting HIF-1; HRE, hypoxia-response element; 2OG, 2-oxoglutarate; PHDs, prolyl hydroxylases; VHL, von Hippel–Lindau protein. Reprinted with permission from Elsevier (149), Copyright (2008).

transactivation domains, a C-terminal transactivation domain (CAD, CTAD) and an N-terminal transactivation domain (NAD, NTAD) (Fig. 6). Bound to DNA, HIF recruits transcriptional coactivators CBP/p300 (Fig. 7), steroid receptor coactivator-1 (nuclear receptor coactivator-1, SRC-1), and transcription intermediary factor 2 (TIF-2), linking HIF and the basal transcriptional machinery to form an initiation complex. As discussed in the previous section, CPB/p300 has also HAT activity, which functions in chromatin remodeling, facilitating the accessibility of HIF to target genes promoters. NAD and CAD differ in the mode of their oxygen regulation. While relatively minor oxygen dependence was observed in the regulation of NAD, regulation of CAD was found to be highly dependent on oxygen. It has been found that CAD is the major transactivation domain mediating the regulation of most HIF target genes. However, the regulation of a subset of HIF-regulated genes was found to be exclusively NAD dependent [reviewed in Ref. (197)]. A recent report demonstrates that the HIF-α TADs, particularly the NTAD, confer HIF target gene specificity, by interacting with additional transcriptional cofactors (127). Activity of the CAD is regulated by hydroxylation of a conserved asparaginyl residue by the asparaginyl hydroxylase, FIH1, which, like the PHDs, is a member of the Fe^{2+} and 2-oxoglutarate (2-OG)-dependent dioxygenase family. Under normoxia, when oxygen is available, FIH1 hydroxylates a conserved asparaginyl residue within the HIF-1α and HIF-2α CADs. The oxygen-dependent hydroxylation of this single asparagine residue leads to a conformational change that prevents the recruitment of the coactivator p300/CBP, resulting in the inhibition of HIF transcriptional activity. However, under hypoxia, CAD hydroxylation is impeded, through the inhibition of FIH1 activity. Hence, hypoxic conditions facilitate CBP/p300 recruitment and thereby promote HIF transactivation activity. Hydroxylation of the single asparagine residue by the FIH1 acts as a molecular switch, allowing the repression of the CAD in normoxia, and its derepression in hypoxia, enabling HIF-regulated gene expression in response to shortage in oxygen supply [reviewed in Refs. (145, 146, 197, 297)].

Thus, HIF activity is regulated by an exceptionally tight, two-level regulatory mechanism in which HIF-α stability is regulated through O_2-dependent proline hydroxylation by PHD (PHD-VHL-proteasome) and its transcriptional activity through the action of O_2-dependent aspargine hydroxylation by FIH1. Both the PHDs and FIH1 are dioxygenases, which during the hydroxylation reaction use both atoms of molecular oxygen, one in the oxidative decarboxylation of 2-oxoglutarate, yielding succinate and CO_2, and the other in the oxidation of the asparagine residue in HIF-α. It has been reported that HIF hydroxylases are present in the cell in limiting amounts and that the intracellular physiological concentration of oxygen is below the apparent K_m in this reaction. Thus, hydroxylases have the capacity to respond to physiological-relevant changes in intracellular oxygen concentrations, linking changes in molecular oxygen concentrations to the regulation of HIF [reviewed in Refs. (91, 146, 197, 298)]. As discussed below, oxygen sensing by the hydroxylases may also proceed via an indirect process mediated through the generation of ROS in the mitochondria under hypoxic conditions (18).

Recently, a functional link was demonstrated between hypoxia and microRNA (miRNA) expression. A microarray-based expression procedure revealed that specific spectra of miRNA are induced in response to low oxygen, some of which were shown to be HIF-dependent. A recent report, using differential tagging, with isobaric tag for relative and absolute quantitation (iTRAQ), followed by liquid chromatography and tandem mass spectrometry (MS) analysis, describes the negative regulation of HIF-1α by miRNA (325). Observations reveal that several hypoxia-regulated miRNA are overexpressed in human cancers, and this indicates their potential role in tumorigenesis [for a recent review on the role of microRNA in the regulation of hypoxic response, see Ref. (179)].

Another level of regulation of gene expression is provided by epigenetic mechanisms. It has been found that oxygen levels can regulate expression of genes through chromatin remodeling (for further discussion see section II.B.6). Several recent studies indicate that expression of angiogenic genes in response to O_2 fluctuations can be influenced by such epigenetic mechanisms [reviewed in Ref. (98)]. For example, hypoxia decreases acetylation of the promoter regulating RUNX3, an inhibitor of angiogenesis, through upregulation of HDAC1, thereby stimulating angiogenesis. Hypoxia induces HDAC1 and HDAC2 protein phosphorylation both in the presence and in the absence of IL-1β (278). Using TBB, an inhibitor of CK II, it was shown that CK II was required for hypoxia-induced HDAC activation. Hypoxic activation of HDAC1/2 also leads to silencing of the *VHL* gene, resulting in stabilization of HIF-1α and angiogenesis (278). Hypoxia-mediated activation of selected genes resulting from histone modifications has been reported. Chromatin immunoprecipitation analyses showed hypoxia-mediated histones modification that correlates with transcriptional response to hypoxia. The promoter region of some hypoxia-inducible genes (e.g., vascular endothelial growth factor [VEGF]) demonstrates decreased demethylation during hypoxia, which could facilitate HIF binding and gene transcription (143). Expression of a group of 2-oxoglutarate-dependent dioxygenases, including Jumonji C-domain, which contains histone demethylase activity, is upregulated by HIF-1α during hypoxia (279). HDAC inhibitors were also found to impair angiogenesis (235) [recently reviewed in Ref. (98)].

3. The role of redox signaling in the regulation of HIF function

a. The source of ROS functioning in the regulation of HIF. A large body of evidence, accumulated during the last two decades, suggests that ROS modulate HIF activity, although the sources and mechanisms of ROS generation are still controversial. H_2O_2 and NO donors were found to stabilize HIF, and genetic and pharmacological interventions that affect ROS generation have been shown to affect the accumulation of HIF-α [reviewed in Refs. (39, 160)]. Brunelle *et al.* investigated the type of ROS required for hypoxic stabilization of HIF-1α protein in genetically manipulated cells that overexpress zinc superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2), GPX1, or catalase. These experiments revealed that cells overexpressing SOD1 or SOD2, which catalyze the breakdown of O_2^- into H_2O_2, did not alter HIF-1α stabilization under the hypoxic conditions used (1.5% O_2, 93.5% N_2, and 5% CO_2), whereas overexpression of GPX1 or catalase, which converts H_2O_2 into water, decreased HIF-1α stabilization under hypoxia, indicating that hypoxic stabilization of HIF-1α requires H_2O_2 and not O_2^- anion (40).

Under normoxia, HIF-1α is responsive to a variety of nonhypoxic stimuli, including various growth factors, cytokines, hormones, coagulation factors, and metal ions. It has been found that many of these nonhypoxic stimuli use ROS in the regulation of HIF-1α and that antioxidants, including ascorbate, vitamin E, NAC, and PDTC, inhibited HIF-1α accumulation and nuclear localization in response to several of these nonhypoxic stimuli [reviewed in Refs. (160, 280)].

As stated above, although ROS have been shown to have an important impact on HIF stabilization and activation, the sources of ROS and the kinetics of their production have been debatable (18, 39, 114, 171, 280, 327). Data accumulated suggest that ROS may be produced either by NADPH oxidases or by mitochondrial components of the electron transport chain. NADPH oxidases have been suggested in various studies as important sources of ROS in regulation of HIF-1α (31, 108, 163). Cytosolic ROS derived from NADPH oxidases were found in several studies to be crucial in regulating the HIF-dependent pathway under nonhypoxic stimuli. Production of ROS by NADPH oxidase 1 (Nox1) activates the HIF pathway (110). ROS production by Nox4 has been shown to be essential for HIF-2α expression and activity in VHL-deficient tumor cells (219). However, a series of studies supported a role for mitochondria, through their electron transfer chain, in the generation of the ROS that are involved in the hypoxic response. It was shown that HIF-α activation was impeded in ρ° cells, which lack functional mitochondria, and in cells treated with inhibitors of the mitochondrial electron transport chain. Further studies demonstrated (see below) (Fig. 8) that in the electron transport chain, complex III is an important source of hypoxic ROS (40, 57, 114). On the other hand, several other reports have challenged these data, demonstrating that in ρ° cell lines HIF-α was stabilized under hypoxic conditions (80). In other studies, the failure to stabilize HIFα under hypoxia in cells with impaired mitochondrial function was interpreted as being the result of reduced mitochondrial oxygen consumption and intracellular O_2 redistribution. According to this proposal, known as the oxygen redistribution model, this led to an increase of O_2 availability and, consequently, the relief of hypoxia, rather than reduced ROS production by the electron transfer chain (80, 116). Recent genetics analyses have demonstrated that mitochondrial electron transport chain is required for stabilization of HIF-1α protein. Cells lacking cytochrome c, and cells in which expression of the Rieske-Fe-S protein, a complex III subunit, was silenced by RNAi, failed to stabilize HIF-1α protein in hypoxia. Pharmacologic and genetic evidence suggests that the ubiquinone (Q) cycle of complex III is the main source of ROS generation during hypoxia that stabilizes HIF-1α protein (40, 49, 50, 113, 218). Complex III can also release O_2^- into the mitochondrial intermembrane space and subsequently into the cytosol (245). Pharmacologic interventions revealed that the Q_o site is responsible for the generation of ROS during hypoxia. Inhibition of electron flux to the Rieske iron-sulfur protein by stigmatellin impedes the generation of ROS and the hypoxic activation of HIF (113). Prevention of electron flux post-cytochrome b, by antimycin A, does not prevent the increase in ROS or HIF activation under hypoxia (50). In accord with these observations it was found, using cytochrome b mutant

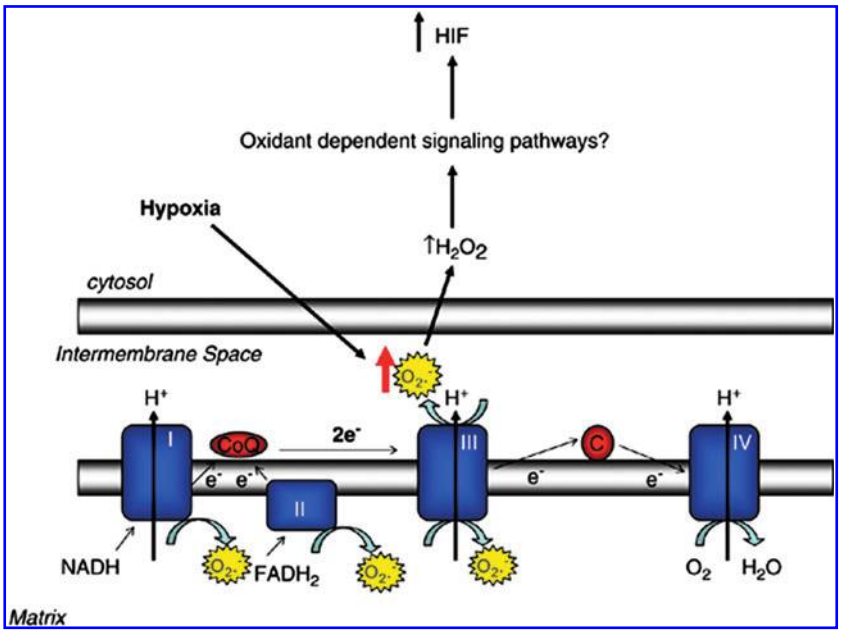

FIG. 8. Mitochondrial electron transport chain generates O_2^- at complexes I, II, and III. Complex I and II generate O_2^- within the mitochondrial matrix. Complex III can generate O_2^- in both the intermembrane space and the matrix. Hypoxia elicits the release of O_2^- from complex III into the cytosol, where it is converted to H_2O_2 to activate oxidant-dependent signaling pathways resulting in the activation of HIF. H_2O_2, hydrogen peroxide. Reprinted with permission from Macmillan Publishers Ltd. (171), Copyright (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

cybrids (19), that hypoxic ROS production was upregulated and HIF-1α was stabilized. A recent study, utilizing the mitochondrial-targeted antioxidant Mito-Q, supports the possibility of a role for ROS in mitochondria signaling to HIF pathway in hypoxia (19).

An NF-κB-regulated mechanism for HIF-1α induction, which is mediated at the transcriptional level, by ROS, was recently reported [reviewed in Ref. (107)], providing a direct link between these two major redox-sensitive transcription factors. Bonello *et al.* have shown in pulmonary artery smooth muscle cells that ROS, which were generated by direct application of H_2O_2 (at the level of 50 μM), overexpression of the NADPH oxidase subunit NOX4, or stimulation of NOX by thrombin, induced HIF-1α transcription via binding of NF-κB to a functional NF-κB-binding site in the HIF-1α promoter (31). van Uden *et al.* demonstrated that NF-κB can directly modulate the HIF-1α pathway. Depletion of NF-κB leads to decrease in HIF-1α mRNA levels. TNFα-induced NF-κB can increase HIF-1α levels, leading to transactivation of target genes in normoxia (349). It was also demonstrated that stimulation of vascular smooth muscle cells by angiotensin-II increases expression of HIF-1α gene and that activation of PKC plays a major role in the increase of HIF-1α gene transcription. Angiotensin-II increases HIF-1α translation by ROS-dependent activation of the PI-3-K pathway (265).

b. ROS effect on HIF–DNA interactions. ROS also modulate HIF-1α activity by affecting the protein directly.

Binding of HIF-1 to DNA requires reducing conditions. The involvement of thiol groups in these interactions was clearly demonstrated in cell extract, where the binding of HIF-1 to DNA could reversibly be abolished by sulfhydryl oxidation. Inhibition of the binding of purified HIF-1 to DNA was observed in the presence of H_2O_2 or diamide (both at the level of 5 mM) (361). Further, the addition of reduced thioredoxin to cell extracts enhanced the binding of HIF-1α to DNA. Consistent with these results is that overexpression of TRX and APE1/Ref-1 significantly potentiated hypoxia-induced expression of a reporter gene in a construct containing the HIF-1α-binding site, and that decreased levels of HIF-1α are detected with an inactive TRX. Studies on the redox-regulated recruitment of the transcriptional coactivators CBP and SRC-1 have established that APE1/Ref-1 potentiates the effect of both coactivators on the activation of hypoxia-inducible promoter by HIF-1α. Moreover, APE1/Ref-1 functionally and physically interacts with the two distinct transactivation domains, NAD and CAD, of HIF-1α. Coexpression of APE1/Ref-1 and thioredoxin in the cells showed that they markedly enhanced HIF-1α transactivation by CAD, not by NAD, in a hypoxia-responsive manner. Thioredoxin, which is present in the cytoplasm under normoxic conditions, was found to translocate to the nucleus, where it was able to interact with APE1/Ref-1 to transfer the redox signal (for further discussion on APE1/Ref-1, see section II.A.3.c). It has also been observed that the redox state of HIF-1α Cys-800 residue was critical for HIF-1α activation (200) [reviewed in Ref. (160)].

c. Mechanism of ROS effect on HIF regulation. ROS regulate HIF via interference with HIF regulatory pathways, including the modulation of the prolyl hydroxylation reactions, performed by the PHD1-3 at proline residues Pro-402 and Pro-564 of HIF-1α and that of the asparagyl hydroxylation, by FIH1 at asparagine residue Asn-803. As described above, both these types of hydroxylases are dioxygenases that require O_2, 2-oxoglutarate, ascorbate, and Fe^{2+} and are affected by changes in the levels of these cofactors. Fe^{2+}, which is lost during the hydroxylation reaction, was proposed to be regenerated in the course of the reaction, through the reduction of Fe^{3+} to the required Fe^{2+} by ascorbate. Hence, accumulation of ROS would affect this process and lead to inactivation of PHD and, consequently, to stabilization of HIF-1 (280). In support of such mechanism are the observations that in JunD-deficient cells, accumulation of ROS in the cell due to lack of expression of JunD-regulated antioxidant pathways leads to increased HIF-1 activation through decreased availability of Fe^{2+} and, consequently, to attenuated activity of PHD and lack of HIF hydroxylation and degradation (103).

4. Involvement of RNS in HIF regulation. The HIF system reveals a linkage between molecular oxygen, O_2^-, and NO in activating or attenuating HIF-mediated responses to hypoxia. It shows that NO and O_2^-, as well as the action of antioxidant enzymes, have a profound effect on HIF-1α stabilization. Studies in different types of cells have shown that NO [applied as 0.5 mM SNAP (166)] stabilizes HIF-1α protein and induces HIF-1α target gene expression under normoxia [for review see Refs. (38, 39, 393)]. An NO-responsive *cis*-element was defined at the HIF-1-binding site (HBS) and an adjacent ancillary sequence (HAS) located immediately downstream within the HRE. It was identified as a common structure of the HRE widely abundant among hypoxia-inducible genes, including VEGF, erythropoietin (EPO), and some genes encoding glycolytic enzyme (167).

Expression of iNOS induces HIF-1α accumulation, demonstrating the role of NO as an intracellular activator for HIF-1α (38). Macrophage-derived NO triggered HIF-1α upregulation in target cells, indicating the intercellular signaling properties of NO in achieving HIF-1α accumulation. These observations suggested that NO functions as an HIF-1 inducer (331, 393). It has been demonstrated that the inhibitory effect of NO on HIF-1α ubiquitination and interaction with pVHL results from its action on the PHDs as targets, which suggests attenuation of prolyl hydroxylation as the underlying mechanism of NO-induced HIF-1α accumulation, in normoxia (233). A recent report describes the normoxic stabilization of HIF-1α by a mechanism that is independent of the oxygen-dependent prolyl hydroxylase-based pathway. It was found that in murine tumors exposed to ionizing radiation, the stimulated generation of NO in the tumor-associated macrophages leads to S-nitrosylation of HIF-1α at the Cys-533 residue of its ODD domain, preventing its degradation. Selective disruption of this S-nitrosylation significantly attenuated the induced stabilization of HIF-1α (187). It has also been demonstrated that pVHL is a target for S-nitrosylation at its Cys-162 residue, which is necessary for the interaction of pVHL with elongin C within the HIF–E3 ligase complex, altering the interaction between pVHL and HIF-1α and decreasing HIF-1α ubiquitination (266).

NO destabilizes HIF-1α under hypoxia and thereby inhibits hypoxia-induced accumulation of HIF-1α. Both ROS and RNS were found to inhibit HIF-1α DNA-binding activity and HIF-1α accumulation under hypoxic conditions (38, 266). Studies in which various intracellular concentrations of NO were generated by the controlled expression of human iNOS revealed that low concentrations of NO caused a rapid decrease in HIF-1α stabilization under hypoxic conditions, whereas high concentrations of NO stabilized HIF-1α under both normoxia and hypoxia. In these experiments NO concentrations of approximately 1 μM generated by iNOS or a dose of 500–1000 μM DETA-NO stabilized HIF-1α, irrespective of the oxygen partial pressure. Lower NO concentration at the range of 400 nM generated by regulating inducible expression of cellular iNOS, or exogenously by ~10-fold lower dose of DETA-NO (50–100 μM) was able to decrease HIF-1α, stabilized by exposure of cells to 3% oxygen (39). Thus, following the oxygen redistribution model, when NO inhibits mitochondrial respiration under hypoxia, it prevents mitochondria from consuming intracellular oxygen, enabling the shifting of oxygen from mitochondria to the PHDs and thereby the continued hydroxylation and degradation of HIF-1α under the hypoxic (but not anoxic) conditions (44, 80). A recent study describes a biphasic effect of NO on the activity of HIF, where high concentrations of NO (250 μM GSNO) prevents HIF hydroxylation, resulting in HIF accumulation and stimulation of expression of its target genes, which include the genes encoding PHD2 and PHD3. This, in turn, leads to downregulation of HIF-1 (22). Another study suggests that NO mediates destabilization of HIF under hypoxia through increased ROS production (44).

In summary, the data presented here demonstrate that ROS/RNS influence the action of HIFs at several different levels: they affect the regulation of HIF-1α synthesis, modify HIF-1α stability or activity directly, or interfere with upstream regulatory signaling pathways. This includes the direct interference in the interactions of HIF-1α with its specific binding elements at the promoters of target genes, through the oxidation of the thiol groups required for the binding of HIF-1α to DNA. Hence, reduction through the action of TRX and Ref-1 significantly enhances the binding of HIF-1α to DNA and the resulting transactivation of target genes. ROS/RNS can also control HIF-1α activity indirectly by affecting the regulation of its synthesis, its stability, and its interaction with transcription coactivators, as well as by interference with regulatory upstream signaling pathways. ROS has been found to modulate the levels of HIF-1α not only under hypoxia, but also under normoxic conditions, in response to various factors and stress stimuli. However, the source of ROS used in hypoxic stabilization of HIF-1 has been debatable for many years. Recent genetics and pharmacological analyses are in support of earlier reports, indicating a major role of mitochondrial electron transport chain in HIF-α stabilization, suggesting that mitochondrial complex III is a major source of ROS for HIF stabilization under these conditions. RNS have been found to affect HIF-1α under both hypoxic and normoxic conditions. High concentrations of NO stabilize HIF-1α under normoxia. However, during hypoxia, low concentrations of NO enhance the destabilization of HIF-1α.

D. Nuclear factor E2-related factor 2

1. Nrf2 structure and function. Exposure of mammalian cells to oxidative stress results in a rapid cellular response, which includes the coordinated expression of a series of antioxidant gene products that effectively restore redox homeostasis. Nrf2 is the key transcription factor that functions in the regulation of this antioxidant response. Nrf2 is induced by both endogenous activators, such as ROS and RNS, and exogenous agents, such as heavy metals and electrophilic xenobiotics [recently reviewed in Refs. (62, 65, 175, 185, 191, 192, 215, 247, 261, 350)]. On the basis of microarray and genetic analyses, it is estimated that Nrf2 modulates transcription of about 200 genes whose encoded protein products play an essential role in cellular defense against oxidative stress. They function as antioxidants, phase II detoxification enzymes, heat-shock proteins, proteasomal components, and GSH synthesis enzymes, providing multiple layers of protection from cellular insults. Few representative examples of genes expressed under Nrf2 transcriptional regulation are NADPH Quinone oxidoreductases (NQO), aldo-keto reductase (AKR), GSH synthetase (GS), GST, PRX1, thioredoxin reductase (TRXR), TRX, heme oxygenase-1 (HO-1), manganese superoxide dismutase (MnSOD), and catalase. Nrf2 also functions

in cell survival, as an inhibitor of Fas-induced apoptosis, probably through the Nrf2-dependent increase of intracellular levels of GSH (175, 192).

Nrf2 is a member of the CNC family of transcription factors (132), which contains a C-terminal bZip structure. It consists of six conserved protein domains, known as Nrf2–ECH homology (Neh) domains (Fig. 9). Of these, the Neh1 is the CNC–bZIP domain. It is involved in the heterodimerization of Nrf2 with the bZIP domain of a group of small Maf proteins [recently reviewed in Ref. (26)], and with the binding of the heterodimer protein to an antioxidant/electrophile-responsive element (ARE/EpRE). The Neh2 domain is an important redox-sensitive regulatory domain containing ETGE and DLG amino acid motifs that are required for the binding of Nrf2 to its cytosolic repressor Keap1, which is involved in the proteasome-mediated degradation of Nrf2. The Neh2 domain also contains one of the three nuclear localization sequences that have been identified in Nrf2 (330). Neh3, Neh4, and Neh5 domains are involved in the Nrf2 transactivation activity, and the Neh6 domain is thought to be involved in Nrf2 degradation [for review see Ref. (192)].

Under redox homeostasis conditions, Nrf2 is located mainly in the cytoplasm, where its activity is sequestered by its complex with Keap1 (133). Keap1 interacts with cytoskeleton filamentous actin and with myosin VIIa, and may act as a cytosolic anchor of Nrf2 (152). Similarly to other proteins of the bric-a-brac, tram-track, and broad complex (BTB) family, Keap1-functions as a substrate adaptor for a cullin-dependent E3 ubiquitin ligase complex (69, 174) and targets Nrf2 for ubiquitination of lysine residues, within the Nrf2 Neh2 domain, and subsequent proteasomal degradation (227, 376, 385) (Fig. 10).

2. Redox regulation of Nrf2

a. Activation of Nrf2 in the cytoplasm. Nrf2 contains two Keap1-binding motifs in the Neh2 domain, an ETGE motif and a DLG motif that can mediate the binding of two Keap1 molecules. Binding of Keap1 is through an overlapping contact of its degradation domain with these two motifs in Nrf2 (Fig. 10) (203, 228, 264, 337). Isothermal calorimetry measurements revealed a two-phase binding in the interaction of Keap1 and Nrf2. Keap1-binding affinity to the ETGE motif was found to be two orders of magnitude higher than its binding affinity to DLG (337, 339). A model, known as the "hinge and latch" (337, 338), describes the interactions between a Keap1 dimer and an Nrf2 molecule (203, 337). It proposes that the high-affinity ETGE motif provides the hinge, through which the bound Nrf2 can move in space relatively freely, while its concomitant binding through the low-affinity DLG motif provides the latch, which restricts Nrf2 movement at the optimal positioning for lysines ubiquitination (228, 337). The model suggests that when challenged by oxidative stress, resulting from accumulation of ROS or RNS, binding of Keap1 to the DLG latch is lost. Since Keap1 binding to the hinge alone is insufficient for providing the proper spatial positioning of target lysines, their ubiquitination is impaired, and thereby targeting of the Keap1-bound Nrf2 to proteasomal degradation is impeded (87, 175, 385). Saturation of Keap1 by bound Nrf2 enables newly synthesized free Nrf2 to translocate to the nucleus and regulate the antioxidant response, via ARE-regulated target genes (Fig. 10).

Keap1 is considered the key regulator of Nrf2 signaling. It is a cysteine-rich protein containing 27 cysteine residues in the human protein (hKeap1) and was considered as a putative candidate for a redox sensor in the regulation of Nrf2 (77). The role of Keap1 cysteine residues in the regulation of Nrf2 has been studied using chemical modification, site directed mutagenesis, and mass spectrometry analyses to assess the relative reactivities of Keap1 cysteines toward different electrophiles (77, 87, 126, 208). It was found that nine of the cysteines in the mouse Keap1 are flanked by one or two basic amino acid residues, a location that was predicted to support a decreased pKa value and increased redox reactivity. However, data accumulated on sulfhydryl modifications suggest that the reactivity of Keap1 cysteines is also affected by the complexity of their three-dimensional context in the protein. As suggested by fluorescence analysis, chemical modifications of cysteine residues result in a profound conformational change of Keap1, as indicated by the substantial quenching of intrinsic tryptophan fluorescence measured (78). On the basis of chemical modification analyses, certain cysteines in Keap1 were considered as preferable targets for electrophilic inducers of Nrf2 and antioxidant enzymes (79). Mutational analyses have revealed the role of several of these cysteine residues

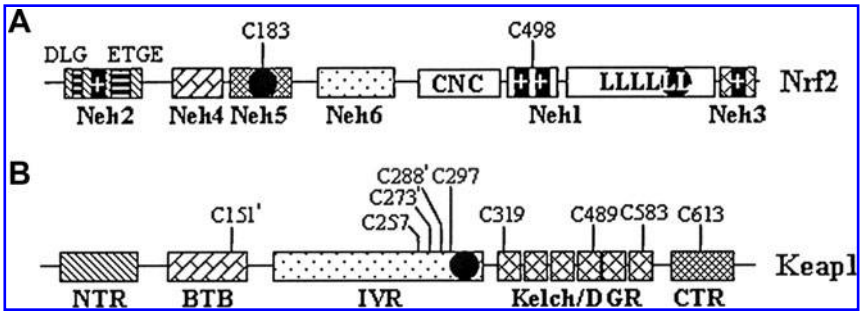

FIG. 9. Schematic drawings of the molecular structure of Nrf2 and Keap1. (**A**) The Nrf2 molecule consists of cap 'n' collar-basic leucine zipper (LLLLLL) Neh1 domain, Keap1-binding Neh2 domain (DLG and ETGE motifs are Keap1-binding motifs), Neh3 domain, a tandem of Neh4 and Neh5 TA domains, and a linker Neh6 domain. The NES motifs are designated with filled circles. The bipartite NLS motif located in the basic region (++) is designated by double filled bars. The monopartite NLS motifs (+) located at the amino-terminus and carboxyl-terminus are designated by single filled bars. The positions of putative reactive cysteines (C-183 and C-498) are also illustrated. (**B**) The Keap1 molecule consists of an amino-terminal region (NTR), a BTB region, an IVR, a Kelch/DGR domain, and a CTR. Keap1 also possesses an NES (filled circle) in the IVR domain. Chemically reactive cysteines are illustrated. Functionally important cysteines are labeled (′). BTB, bric-a-brac, tram-track, broad complex; CTR, carboxy terminal region; DGR, double glycine repeat; IVR, intervening region; Nrf2, nuclear factor E2-related factor 2; TA, transactivation. Reprinted with permission from John Wiley & Sons, Inc. (192), Copyright (2009).

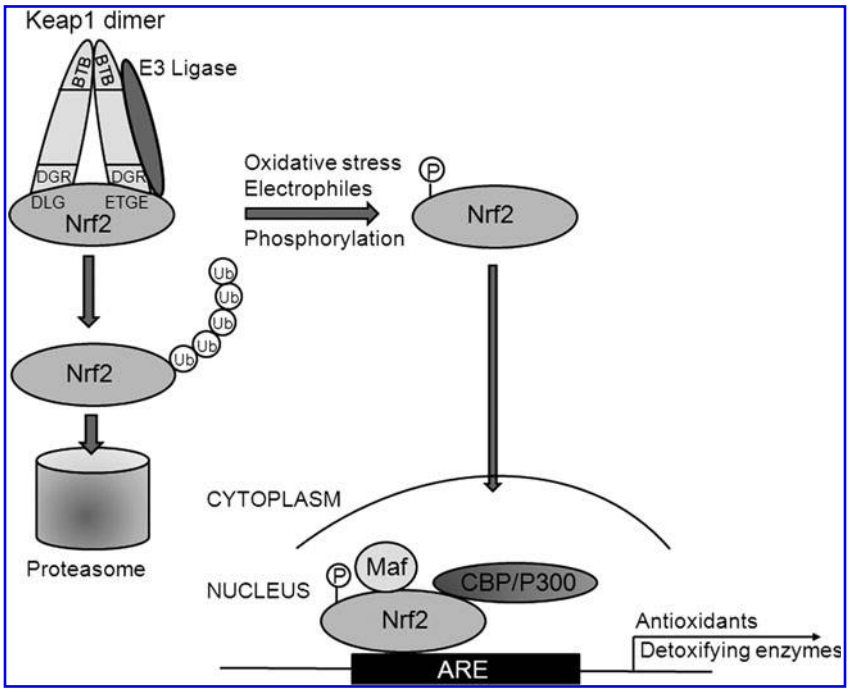

FIG. 10. The Nrf2 signaling pathway. Under basal nonstress conditions, a molecule of Nrf2 is bound to a Keap1 dimer in the cytoplasm. Keap1 links Nrf2 for interaction with the Cullin-dependent E3 ubiquitin ligase complex E3-ubiquitin ligase complex (indicated by "E3 Ligase"). The interactions of the Keap1 DGR domain with the DLG and the ETGE motifs of Nrf2 enable the efficient ubiquitination (Ub) of Nrf2 and its direction for proteasomal degradation. According to the hinge and latch model, as a result of exposure to stressors, such as ROS, RNS, heavy metals, or electrophilic xenobiotics, the binding between Keap1 and Nrf2 through the low-affinity DLG latch is perturbed via the phosphorylation of Nrf2 and/or a conformational change in Keap1 through the modification of cysteine residues, while binding through the high-affinity ETGE hinge is maintained, but Nrf2 is no longer at the correct position to be ubiquitinated and hence its proteasomal targeting is impaired. As a result, Keap1 becomes saturated, which enables the accumulation of free, newly synthesized Nrf2. Stabilized free Nrf2 translocates into the nucleus, where it dimerizes with a small Maf protein, binds to ARE sites at the promoters of Nrf2 target genes, recruits the transcription coactivator, and transactivates expression of target genes. RNS, reactive nitrogen species.

in Keap1 function. Cys-151, in the Keap1 BTB domain, was found to be important in chemical/oxidative stress-stimulated derepression of Nrf2 and prevention of its ubiquitination and proteasomal degradation (385). Cys-273 and Cys-288, located within the Keap1 cysteine-rich domain, are essential for the repression of Nrf2 under basal conditions of redox homeostasis [reviewed in Ref. (175)] and were suggested as targets for electrophilic inducers of Nrf2. It was shown that the mouse Keap1 could form a complex with Nrf2 under reduced conditions (77) and that the Neh2–Keap1 complex could be disrupted by high concentrations of solforaphane (SFN, 10 mM) or bis(benzylidene) acetone (14 μM) (77). It has also been shown that mutating Keap1 cysteines decreases its binding affinity to Neh2 (358). These observations suggested that Keap1 may function as a primary redox sensor that undergoes conformational changes as a result of the modification of cysteine thiols, leading to Nrf2 release and the transmission of the redox signal into the nucleus (192).

Other studies on the relationship between the thiomodification of Keap1 and Nrf2 release argue against such a "direct disruption" model. They indicated that the Nrf2–Keap1 complex is stable under oxidative stress and that thiomodifications affect the generation of Keap1–Nrf2 complex, rather than its dissociation (87) [reviewed in Ref. (192)]. On the basis of these data, Li and Kong (192) raised a question regarding the role of Keap1/Nrf2 dissociation as a relay step in the mechanism of Nrf-2 activation and nuclear translocation. These investigators have further shown that silencing of the endogenous Keap1 activity using siRNA has not altered the tert-butylhydroquinone (tBHQ)-induced nuclear translocation of Nrf2 (193). Further, it was found that Nrf2 nuclear translocation has high redox sensitivity and that the nuclear export signal (NES) located at the transactivation domain (NES_{TA}) motif is redox sensitive. Hence, it has been suggested to function as a redox-sensitive switch that can be turned on and off by oxidative signals, determining the subcellular localization of Nrf2 (192).

These studies led to the proposal of an alternative, Keap1-independent Nrf2 signaling model that describes the mechanism by which Keap1 and Nrf2 sense and transmit redox signals. It proposes that under redox homeostatic conditions, an equilibrium is maintained between Nrf2 synthesis and degradation, in which only a small pool of free Nrf2 (fNrf2) relative to Keap1-bound Nrf2 (kNrf2) is available, and sufficient for basal Nrf2 activity under these conditions. Because fNrf2 molecules have high redox sensitivity and high mobility, they can act immediately as redox probes, whereas kNrf2 proteins are redox inert. Upon exposure to oxidative stress, the redox-sensitive ubiquitination of Nrf2 is impaired, but the preformed Nrf2–Keap1 complexes are not disrupted, and since Nrf2 synthesis continues, the capacity of Keap1 to bind Nrf2 is saturated. In addition, whereas Keap1 is self-ubiquitinated and degraded (386), Nrf2 translation is enhanced (282). Hence, redox conditions determine the size of fNrf2 intracellular pool, which implies that the relative abundance of fNrf2 may determine the actual magnitude of an antioxidant response. In this model, both Nrf2 and Keap1 have high sensitivity for redox signals and these signals are not transmitted from Keap1 to Nrf2. Thus, Keap1 functions as a redox-sensitive gate keeper that regulates the size of the pool of fNrf2 in a redox-sensitive manner (192).

Recently, Clements *et al.* have reported on another possible mechanism of Nrf2 regulation, where the stabilization of Nrf2 is affected by DJ-1 protein, a cancer and Parkinson's disease-associated protein (63), which have a profound effect on the abundance of Nrf2 in the cells. These investigators have found that DJ-1 is essential for Nrf2 stabilization, which is promoted by affecting Nrf2 association with Keap1, and consequently impairs its ubiquitination and proteasomal degradation. Although their experiments demonstrated a strong functional link between DJ-1 and Nrf2, they have not revealed the mechanism by which DJ-1 physically exerts this effect. Coimmunoprecipitation experiments have not detected DJ-1 in physical association with Nrf2, Keap1, or E3 (63).

Another potential regulator of Nrf2, through potential competition of Keap1 binding, could be implied from a report that describes a novel substrate of Keap1. Lo and Hannink (202) have demonstrated that a member of the phosphoglycerate mutase family, PGAM5-L, is a *bona fide* substrate for a Keap1-dependent ubiquitin ligase complex. They have found that the N-terminus of the PGAM5 protein contains the conserved NXE(S/T)GE motif required for binding to Keap1. Coexpression of Keap1 with PGAM5-L significantly increases Keap1-dependent ubiquitination of PGAM5 by a Cul3–Rbx1-dependent E3 ubiquitin ligase complex, decreasing the steady-state levels of PGAM5-L. This decrease in PGAM5-L level can be prevented by inhibition of the 26 S proteasome. Quinone-induced oxidative stress, as well as sulforaphane, inhibits Keap1-dependent ubiquitination of PGAM5. Similarly to Nrf2, PGAM5-L is a substrate for Keap1-dependent ubiquitination and proteasomal degradation, but it displays a significantly slower turnover rate.

b. Nrf2 nuclear translocation and transactivation. Translocation of Nrf2 to the nucleus is of a graded nature, which transmits both the redox signal and its magnitude, reflecting the intensity of the oxidative stress, through the size of Nrf2 flux. Several motifs that are involved in the intracellular localization of Nrf2 have been identified in Nrf2. A redox-insensitive NES is located in the leucine zipper domain (NESzip), a redox-sensitive NES motif is located in the Neh5 transactivation domain (NES_{TA}), and a bipartite NLS is located at the basic region (bNLS) (190, 193). Mutational analyses showed that the Cys-183 residue, located in the Neh5 domain, may mediate the redox response of NES_{TA} (191). Mass spectrometry analysis showed that this cysteine residue is redox reactive (191). It was further suggested that the NES_{TA} motif functions as a redox-mediated conditional NES that can be switched off by oxidants, thus promoting Nrf2 nuclear translocation (191). NLSs were identified also in the N-terminal domain (NLS_N) and in the C-terminal domain (NLS_C) [reviewed in Ref. (192)] (Fig. 9). On the basis of the presence of multiple NLS/NES motifs in Nrf2 and the redox sensitivity of NES_{TA}, it was suggested that the combined activities of multiple NLS/NES motifs determine the subcellular localization of Nrf2, which senses and transmits oxidative signals into the nucleus.

Upon nuclear accumulation, Nrf2 heterodimerizes with a Maf-F/G/K protein (157) whose expression was found to be Nrf2/ARE regulated, providing an autoregulatory loop. Only the heterodimer molecule binds the specific consensus *cis*-element, ARE or EpRE motif, present in the promoter region of genes, whose expression is Nrf2 mediated. Binding of Nrf2 is mediated through its bZip structure and may also be affected by oxidation [in the presence of a 100 μM t-BHQ (27)] of its Cys-506 residue in the DNA-binding domain. Recruitment of the coactivator CBP/p300 links Nrf2 with the transcription machinery and RNA polymerase. It enables the initiation of its transactivation activity and expression of Nrf2-regulated genes [reviewed in Refs. (175, 192)].

In addition to its regulation via the modification of specific thiol groups, the Nrf2 signaling can be modulated directly by phosphorylation [reviewed in Ref. (62)]. It has been reported that the phosphatase inhibitor okadaic acid stimulates Nrf2 nuclear accumulation and transactivation activity, implying that enhanced phosphorylation promotes Nrf2 activity. Indeed, phosphorylation of Nrf2 affects both its stability and localization. In response to oxidative stress Nrf2 is phosphorylated by several pathways, including that of PKC, MAPK, and the PI-3-K pathways. Phosphorylation by protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), CK II, GSK3β/Fyn, ERK, and JNK was documented (28, 135, 136, 151). It has also been reported that the phosphorylation of Nrf2 at Ser-40 by PKC is required for Nrf2 to evade Keap1–Nrf2-mediated degradation, but was not required for Nrf2 stabilization or accumulation in the nucleus and for the transcriptional activation of ARE-mediated gene expression (28). Phosphorylation of Nrf2 in the transcription activation domain by CK II, however, was reported to be critical for nuclear translocation and transactivation (14), as well as for its degradation (276). MAPK pathways were reported to induce Nrf2-mediated gene expression. ERK2, ERK5, and JNK MAPK activate Nrf2, whereas p38 MAPK phosphorylation of Nrf2 was found to increase its association with Keap1 and prevent Nrf2 translocation (62). Expression of MEKK1, TGF-β-activated kinase (TAK1), and ASK1 activated an Nrf2-dependent reporter gene, whereas expression of their mutants impaired activation upon exposure to arsenite and mercury. In a study on the roles of ERK and JNK in the regulation of phenethyl isothiocyanate (PEITC)-induced Nrf2-dependent activity, it was found that phosphorylation of ERK1/2 and JNK1/2 increased. Nrf2 was phosphorylated and translocated into the nucleus (375). Several inducers of Nrf2 upregulate the PI-3-K/AKT pathway. Specific inhibition of PI-3-K/AKT partially prevented H_2O_2-induced translocation of Nrf2 to the nucleus, implying the role of this pathway in the nuclear translocation of Nrf2, in response to oxidative stress (151). It has also been reported that PI-3-K/AKT regulates the rearrangement of actin microfilaments in response to oxidative stress, affecting the translocation of actin-bound Nrf2 into the nucleus. It has been suggested that the regulatory role of PI-3-K/AKT may be due to its inhibitory effect on GSK3β (295). It has been proposed that GSK3β may be involved in the cytoplasmic sequestration of Nrf2 in the absence of ROS, and that PI-3-K/AKT inactivation of GSK3β may contribute to the release of Nrf2 repression in response to oxidative stress (62). Phosphorylation of the Nrf2 Tyr-568 residue by Fyn was reported to be essential for the nuclear export of Nrf2 (135, 136, 295). Mutants in Nrf2 Tyr-568 were not phosphorylated and were found to accumulate in the nucleus, as a result of their loss of capacity to interact with the nuclear export protein Crm1 (135). Cullinan *et al.* (70) have identified Nrf2 as a substrate for phosphorylation by PERK, whose function is essential for cell survival after exposure of cells to ER stress. These investigators have found that PERK-dependent

phosphorylation of Nrf2 triggers dissociation of Nrf2–Keap1 complexes and inhibits their reassociation *in vitro*. Activation of PERK, via agents that trigger the unfolded protein response, promotes the dissociation of cytoplasmic Nrf2/Keap1 and subsequent Nrf2 nuclear import.

The data described here demonstrate that redox signaling controls several levels in the process of Nrf2 activation. At the level of Nrf2 stabilization, oxidative stress affects the interaction between Nrf2 and Keap1, through the multiple reactive Cys residues in Keap1 that are targets for modifications by ROS. This affects Nrf2–Keap1 complexes, the subsequent ubiquitination of Nrf2, and its proteasomal degradation, thereby determining the intracellular pool of free Nrf2. Then, nuclear translocation and nuclear accumulation of Nrf2 are affected by its capacity to sense the redox state and the changes in its amplitude as a result of oxidation, through cysteine residues in Nrf2, affecting its nuclear import/export balance and nuclear translocation rates. Oxidants and electrophiles can further affect the Nrf2 nuclear accumulation through the redox sensitivity of some of the pathways (see above) that promote the phosphorylation of Nrf2, affecting its binding to Keap1, its proteasomal degradation, and its nuclear translocation. Then, while within the nucleus, the heterodimerization of Nrf2 with Maf, whose expression is under Nrf2/ARE-mediated regulation, presents an apparent, redox-affected, autoregulatory feedback loop. Finally, the potential direct effect on the DNA-binding capacity of Nrf2, through the redox-mediated modification of a cysteine residue at Nrf2 DNA-binding domain, was also reported. In conclusion, although other pathways were reported to be involved in Nrf2 control, redox signaling is the pathway that plays the major role in the regulation of Nrf2 (Fig. 10).

E. p53 tumor suppressor protein

1. p53 structure and function. p53 is a sequence-specific transcription factor that serves as a potent tumor suppressor. Approximately half of human cancers were found to have inactivating mutations of p53 (TP53 in human). p53 has been named "the guardian of the genome" because it plays a central role in coordinating the cellular responses to a broad range and levels of cellular stress factors, leading to apoptosis, cell cycle arrest, senescence, DNA repair, changes in cell metabolism, or autophagy [for recent reviews on p53 see Refs. (16, 46, 66, 93, 111, 121, 178, 194, 290, 357, 367, 388)]. There are hundreds of p53 DNA-specific binding sequences located close to promoters of target genes in the human genome. Their consensus sequence of p53-binding element consists of two inverted pentameric palindromes with the pattern 5′-RRRC(A/T)|(A/T)GYYY-3′ (R, purine; Y, pyrimidine) separated by 0–13 base pairs (88). Affinities of p53 for specific p53-responsive elements differ widely, suggesting the involvement of additional parameters in the regulation.

The factors that influence the ability of p53 to respond specifically to diverse range and levels of stress signals have been a focus of many studies. Various posttranslational modifications of p53 influence its promoter selection and consequently affect cell fate, through the induction of apoptosis, cell cycle arrest, or other p53-dependent pathways (Fig. 11). It has been suggested that these modifications induce alternate p53 conformations that affect the recognition of different responsive elements. Among these numerous modifications, phosphorylation of the p53 Ser-46 residue was reported to selectively enhance the apoptotic but not the cell cycle-arrest function of p53; acetylation of the K-120 residue, in response to severe DNA damage, preferentially enhances transcription of proapoptotic genes, but not p53-growth arrest, whereas the competition between acetylation and ubiquitination at the K-320 residue directs cell-fate toward apoptosis or growth arrest, respectively [recently reviewed in Refs. (16, 74, 184)]. There are several structural determinants that were proposed to affect promoter discrimination. Some responsive elements (such as the promoter regions for the p53 target genes Mdm2, GADD45, p21, 14-3-3σ, and KARP-1) were found to exist in a constitutively open, nucleosome-free conformation, which may promote p53 activation by facilitating higher accessibility to these sites (33). Other studies provided evidence that the complex interactions of p53 with DNA are strongly dependent on the dynamics of DNA structure, including the differential capacity of distinct p53-responsive elements to adopt B versus non-B-DNA conformation (165). A recent study proposes a possible correlation between the nature of the higher-order palindrome, generated within the binding sequence, and the functional role of the elements in positive or negative regulation (209). The different binding affinities displayed in the interactions of p53 with its different responsive elements emphasize the potential major effect of p53 levels on promoter choice and consequently on the cell fate. A tendency to favor the activation of cell growth arrest pathway has been reported at low p53 levels, versus the triggering of apoptosis pathways under high cellular levels of the protein, which may be in accord with the relative delayed induction of the later pathways, following stress stimuli (184, 323). The importance of sequence-specific DNA binding is emphasized by the observations that many of tumor-associated p53 mutations occur within its DNA-binding domain (141, 142, 367). Nevertheless, although transactivation of target genes regulated by p53 is an essential feature of each stress response pathway, some effects of p53 have been suggested to be independent of its transcription activity (357).

p53 is active as a tetramer containing four identical protomers of 393-residues [for recent reviews of p53 structure see Refs. (142, 255)]. Its N-terminal region consists of a transactivation domain (TAD), a proline-rich region (PRR), a DNA-binding central core domain, a tetramerization domain, and a lysine rich C-terminal domain. TAD has an intrinsic disordered structure that facilitates the high specificity binding of diverse p53-interacting proteins (83), such as components of the transcription machinery (76), the transcriptional coactivator p300/CBP (329), and the negative regulators Mdm2/Mdm4(x) (or the human homologs Hdm2/Hmd4(x)) (220, 299). The Mdm2-binding region partially overlaps with that of the transcriptional coactivator p300. Hence, binding to p300 would protect p53 from binding of the negative regulators Mdm2 and Mdm4, whereas binding of Mdm2 would prevent p53 interaction with the p300 coactivator. Their competition for the p53 N-terminus will be affected by the differences of their binding affinities and the further modulation by post-translational modifications (142).

The p53 TAD region is followed by the central core DNA-binding domain (DBD) that mediates p53 sequence-specific DNA binding. The structure of the core DBD was solved both in the free unbound form and in complex with DNA and interacting proteins. The structural studies demonstrated that

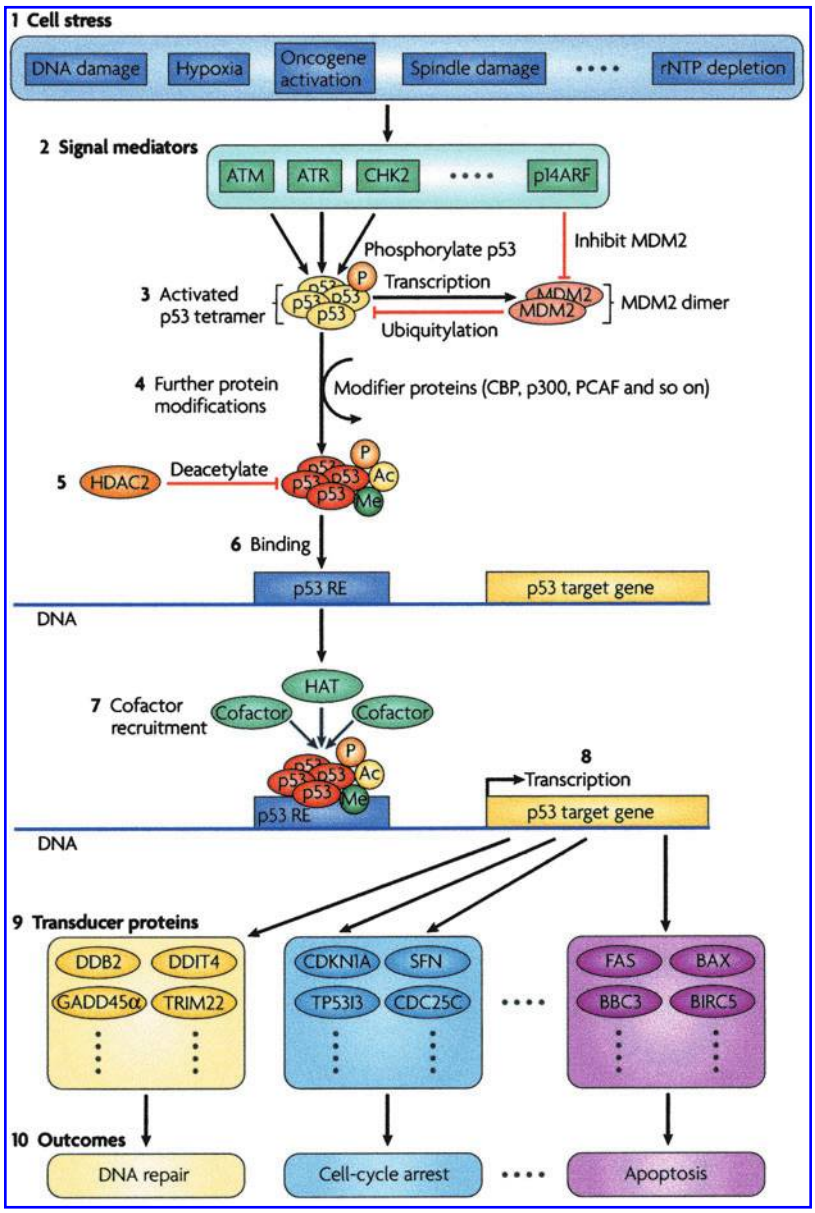

FIG. 11. A model describing mechanisms in p53 activation and regulation of downstream targets. Step 1: Cells undergo stress, which can lead to cancer. Step 2: Signal mediator proteins activate p53 by phosphorylating certain residues or inhibiting ubiquitination by MDM2. Step 3: Both processes increase the half-life of p53 by inhibiting ubiquitination, which leads to higher levels of p53. Step 4: Further p53 modifications by acetyltransferases (CBP, p300, and PCAF) and methyltransferases (SET9) can further stabilize the p53 protein and increase site-specific DNA binding. Step 5: HDAC2 can inhibit p53 binding to DNA by deacetylating the protein. Step 6: The p53 tetramer binds to a p53 RE to regulate transcription of a nearby gene. Step 7: p53 also recruits cofactors such as HATs and TAFs. Step 8: In this example, p53 mediates transactivation of its target gene, but p53 can also mediate transcriptional repression. Step 9: The p53 protein transactivates many genes, the protein products of which are involved in various pathways. Step 10: The most important pathways involved in tumor suppression that are activated by p53 lead to DNA repair, cell cycle arrest, senescence, and apoptosis. HATs, histone acetyltransferases; RE, response element; TAFs, TATA-binding protein-associated factors. Reprinted with permission from Macmillan Publishers Ltd. (290), Copyright (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

the binding surface consists of two structural motifs that bind to target DNA. One half forms loop-sheet-helix motif, which docks to the DNA major groove, and the other forms two large loops, which are stabilized by a zinc ion, which is tetrahedrally coordinated by a histidine and three cysteine side chains (Cys-176, His-179, Cys-238, and Cys-242) [reviewed in Ref. (142)]. It has also been reported that the conformation of this domain remains relatively unchanged after the binding to DNA (125). Four p53 core domains bind to a p53-binding sequence element in the DNA in a highly cooperative manner (366). The key residues in the p53 core–DNA interface that are in a direct contact with a DNA half-site are Lys-120, Ser-241, Arg-248, Arg-273, Ala-276, Cys-277, and Arg-280 (59, 168).

The central core DBD region is connected by a flexible linker to a short tetramerization domain that regulates the tetramerization of the protein (352). Two monomers form a primary dimer and then two such dimers associate, through their helices, to form a four-helix bundle tetramer (142). The C-terminal domain also contains a region rich in basic amino acids (mainly lysines) that binds DNA nonspecifically. It enables p53 to bind nonspecific DNA targets, such as mismatched DNA, double-strand breaks, single-stranded DNA, and Holliday junctions (307).

2. Regulation of p53 activity

a. Activation of p53. In unstressed cells, under normal cell growth conditions, the level of p53 protein is kept low through regulation of its protein stability [for recent review of p53 regulation see Refs. (16, 178, 336)]. Under these growth conditions, Mdm2 binds to p53, blocks its interaction with transcriptional coactivators, and ubiquitinates p53, targeting it for proteasomal degradation [recently reviewed in Refs. (66, 319)]. Stress-related signals activate and stabilize p53 (Fig. 11) primarily through posttranslational modifications. DNA damage, as well as other types of stress, activates damage-responsive kinases that phosphorylate p53 N-terminal Ser-15 and Ser-20, by various protein kinases, including DNA-dependent protein

kinase (DNAPK), ATM, and ataxia telangiectasia RAD3-related kinase (ATR) and their downstream kinases checkpoint kinase-2 (Chk2) and checkpoint kinase-1 (Chk1). These modifications and the resulting conformational changes reduce the affinity of p53 to Mdm2, stabilizing p53 by disrupting its complex with Mdm2, promoting acetylation and phosphorylation of the C-terminus, and enabling its tighter association with coactivators, such as p300. The six C-terminal lysines of p53 are targeted by Mdm2 for ubiquitination, and are acetylated by CBP/p300 (46), and the competition between ubiquitination and acetylation of the lysine residues is thought to affect p53 stability. p53 acetylation levels are markedly enhanced in response to stress, promoting p53 stabilization and activation (36). As p53 regulates expression of its inhibitor Mdm2, the observation that both phosphorylation and acetylation of p53 can inhibit Mdm2–p53 interactions indicates an important role of posttranslational modifications in affecting the p53/Mdm2 feedback loop.

There are several proteins that interact with Mdm2 and function as its regulators. First, the tumor suppressor ARF was found to interfere with the Mdm2–p53 interaction, and consequently to stabilize and activate p53 (311). Mdmx (Mdm4) is an important Mdm2 regulator that, similarly to Mdm2, functions as a negative regulator of p53, by binding to p53 TAD and inhibiting its transcriptional activity via interference with its ability to recruit coactivators and to interact with the basal transcription machinery. Mdmx binds Mdm2 through their C-terminal RING domains and stabilizes Mdm2 by interfering with its auto-ubiquitination activity [reviewed in Ref. (220)]. Another regulator of Mdm2–p53 interactions is the transcription factor YY1, which was found to interact directly with Mdm2 and p53 and facilitates the Mdm2–p53 interaction, promoting the ubiquitination of p53 *in vivo* and *in vitro* (320). It has also been reported that binding of the ribosomal proteins L5, L11, and L23 to Mdm2 inhibits Mdm2 function and plays a crucial role in p53 activation upon ribosomal stress (72, 204). Mdm2 is also positively and negatively regulated by phosphorylation and acetylation [reviewed in Ref. (234)]. Several RNA-binding proteins, including HuR (Hu antigen R), ribosomal protein L26 (RPL26), and nucleolin, have been shown to increase p53 levels after DNA damage, through promotion of p53 translation (326).

b. DNA binding and transactivation. Binding of stress-activated, posttranslationally modified p53 to specific response elements of target genes is required to initiate its transactivation activity. Sequence-specific DNA binding is carried out via the p53 core DNA-binding domain, whereas the C-terminal domain was thought earlier to function as an allosteric modulator whose posttranslational modifications (such as phosphorylation, ubiquitination, methylation, sumoylation, neddylation, and acetylation) modulate the sequence-specific DNA binding by the central core domain and coactivators recruitment (36, 46). Recent observations suggest that both the core DNA-binding domain and the C-terminal domain of p53 possess DNA-binding activities. It was also found that a significant fraction of p53 is bound to DNA in unstressed cells and that only a small fraction of it is bound at p53 consensus sequence elements (47, 147). Moreover, p53 was found bound at its targets' binding sequence, under nonstressed conditions, while displaying no transactivation at these sites (147, 323) [recently reviewed in Ref. (178)]. A model that describes the binding of p53 to its targets in genomic DNA proposes a plausible explanation for these observations. It suggests that two separate DNA-binding domains with distinct binding properties function in p53. A facilitated search for the target sequence is promoted by the p53 basic DNA-binding domain [reviewed in Ref. (201)], where p53 does not bind to its specific binding site directly, but rather through two equilibria, first binding randomly to nonspecific sequences in the genomic DNA, followed by sliding on the DNA to the specific binding sites in the genome.

As discussed above, in the case of other transcription factors (section II.B.6), histone acetyltransferases (HATs) and HDAC also provide an important layer of p53 regulation (36), through the manipulation of chromatin structure. It has also been found that components of the chromatin remodeling SWI/SNF complex interact with p53, enhancing p53-mediated cell growth arrest [reviewed in Ref. (16)]. In addition, it has been reported that p300, together with JMY, a p300 cofactor, is recruited to p53 in response to genotoxic stress, and significantly enhances the apoptotic response (66).

3. The role of redox signaling in the regulation of p53

a. The effect of p53 on energy metabolism and the intracellular level of ROS. ROS and p53 play central roles in cellular signaling pathways, and their interactions result in major effects on cell physiology. p53 function affects the levels of cellular ROS, and ROS, in turn, affects the selective transactivation of p53-regulated genes. p53 functions in the modulation of intracellular ROS levels by both pro and antioxidant activities, each contributing to its function as tumor suppressor [recently reviewed in Ref. (198)].

Recent studies demonstrate that p53 regulates energy metabolism and its resulting ROS generation by modulating the transcription of p53 target genes that control mitochondrial respiration, glycolysis, and the pentose phosphate shunt [recently reviewed in Refs. (21, 198, 259, 341)]. SCO2 (synthesis of cytochrome *c* oxidase 2), a protein that regulates the cytochrome c oxidase complex and is essential for mitochondrial respiration, is encoded by a p53-target gene. In the absence of functional p53, the deficiency in SCO2 results in lower oxygen consumption by mitochondrial respiration, which leads to a metabolic shift to glycolysis for the production of energy, a phenomenon known as the Warburg effect. The shift to glycolysis results in a reduction of the level of ROS, generated by mitochondrial respiration, suggesting that a shift in metabolism from respiration to glycolysis is being used as a strategy to decrease oxidative stress by lowering the level of ROS production [reviewed in Refs. (21, 198)].

p53 affects glucose metabolism at several levels. It can affect glucose uptake by repressing transcription of the genes *GLUT1* and *GLUT4*, which encode glucose transporters. In other cases it modulates expression of p53 target genes that encode glycolytic enzymes, including phosphoglycerate mutase (PGM) and hexokinase II (HK). These *trans*-activation and *trans*-repression activities couple p53 to the regulation of energy metabolism, affecting intracellular ROS levels [recently reviewed in Refs. (176, 259)]. Another pathway used by p53 to lower the levels of cellular ROS and promote the survival of cells undergoing mild stress is through the regulation of the p53-induced gene, TP53-induced glycolysis and apoptosis regulator (TIGAR) (20). TIGAR encodes for the

enzyme fructose-2,6-biphosphatase that reduces the level of fructose-2,6-bisphosphate in the cell, resulting in inhibition of glycolysis. Consequently, glucose is directed to the pentose phosphate shunt, whose activation leads to the production of the NADPH, which is used for generation of reduced GSH. GSH, in turn, functions as an antioxidant that promotes the scavenging of ROS, enhancing genomic stability and thereby mediating the tumor suppression effect of p53 (20).

b. ROS effect on p53 activity. p53 is a redox-sensitive transcription factor, a zinc-binding protein, that contains 10 cysteine residues. Beyond the direct effects of redox on p53 structure and function, as described below, various redox systems can regulate the function of p53 through indirect oxidative modifications and through its interacting partners such as Mdm2, which provides another level of redox regulation of p53 (122).

Multiple reports suggest that p53–DNA binding ability and binding selectivity can be modulated by ROS and RNS. The data accumulated suggest that ROS signaling affects p53 activity on several levels of p53 structure and function. This includes indirect effects, through ROS regulation of pathways that function in the posttranslational modification of p53 and the interaction of the p53 pathway with other signaling networks, as well as the direct effect of ROS on the structure and function of p53. Among the multiple posttranslational modifications, p53 phosphorylation, which affects its stabilization and activity, is mediated by several redox-sensitive protein kinases, including MAP kinases ERK, JNK, p38α (321), and ATM (180), which have been shown to activate p53 in response to various stress signals, including ROS signaling (Fig. 12).

c. Redox regulation of p53 DNA-binding activity. p53 contains several reactive cysteine residues within its DNA-binding domain, rendering it redox sensitive. Binding of p53 to DNA *in vitro* requires the presence of thiol-reducing agents and is impaired by thiol oxidants [reviewed in Ref. (117)]. Three cysteine residues in human p53, Cys-176, Cys-238, Cys-242, and a histidine residue, His-179, coordinate the binding of a zinc ion, which stabilizes the loop/helical structure of the core domain (59). Zinc binding is essential for the stabilization of p53, and its exposure *in vitro* to metal chelators, such as EDTA or orthophenanthroline, or *in vivo* to TPEN,*N*,*N*,*N*^{9},*N*^{9}-tetrakis(2-pyridymethyl) ethylenediamine, results in a rapid switch of p53 to an unfolded form and loss of DNA-binding activity [reviewed in Ref. (232)]. It was demonstrated that oxidation by H_2O_2 (5 mM) inhibits the binding of purified p53 to its specific binding sequence, but has no effect on its nonspecific binding to DNA *in vitro*, and that in H_2O_2 (50 μM-1 mM)-treated cells in culture, the lack of specific DNA binding inhibits p53 capacity to transactivate a target gene (272). Mutagenesis of the cysteine residues that are involved in the binding of Zn^{2+} ion leads to loss of specific DNA binding, whereas substitution of Cys-124, Cys-135, Cys-141, and Cys-277, located in the loop-sheet-helix region of the DNA-binding domain of p53 with serine residues, modulates murine p53 affinity to DNA, suggesting that the latter group of cysteines may regulate the structural dynamics of the DNA-binding domain (117). Mass spectrometry of GSH-modified p53 protein identified Cys-124, Cys-141, and Cys-182 residues, which are located in a negatively charged microenvironment, as the sites of glutathionylation. S-glutathionylation of either Cys-124 or Cys-141, which were located by molecular modeling to the dimer interface, inhibits p53–DNA association and interferes with the protein dimerization (322, 351). Cys-277 is exposed at the protein surface and donates a hydrogen bond to bases in the major groove of the DNA (59). Oxidation of Cys-277 would induce conformational changes at the DNA-binding surface (117). Studying the structural elements that determine the specificity of promoter selection by p53, Buzek *et al.* have reported on a mechanism by which the redox state of p53 regulates its differential affinity to distinct responsive elements (43). Their studies revealed that changes in the redox state of Cys-277 allow p53 to discriminate between individual responsive elements, according to their sequence, demonstrating that oxidation of Cys 277 decreases p53 binding to GADD45 but not to p21$^{WAF1/CIP1}$. Using site-directed mutagenesis, these investigators showed that this differential affinity is dependent upon the presence of cytosine at position 3 of the responsive element pentamers and on p53 redox state, linking ROS directly to p53 specificity of function (43).

The physiological role of redox regulation in the control of p53 function is demonstrated through the regulatory function of the redox proteins TRX, TRXR, and APE1/Ref-1 in the control of p53 activity (function of APE1/Ref-1 is discussed in section II.A.3.c). In earlier studies, Ueno *et al.* have demonstrated that TRX enhances the DNA-binding activity of p53 *in vitro* and significantly potentiates APE1/Ref-1-mediated p53 binding. These investigators have also shown in transfection experiments that expression of TRX enhanced p53-dependent expression of p21 and further intensified APE1/Ref-1-mediated p53 activation (347). Further studies, using functional expression of mammalian p53 in yeast, revealed the essential function of TRX and TRXR in the transactivation activity of p53, demonstrating the requirement for reduction of disulfide bonds in p53 (117, 301). As described above, APE1/Ref-1 is a multifunctional protein possessing both DNA repair activity of AP endonuclease 1, and transcriptional regulatory activity of Ref-1, functioning in various pathways of cellular response to oxidative stress [recently reviewed in Refs. (12, 328)]. In addition, it has been previously indicated that through its dual function, APE1/Ref-1 may provide a link between two pathways of p53 regulation, DNA-damage sensing and redox modulation (117). Recent studies have demonstrated the involvement of p53 in the regulation of APE1/Ref-1. Zaky *et al.* have shown that p53 regulates APE1/Ref-1 expression after genotoxic stress (381). They have found that wild-type p53, but not its null mutant, is functioning in the cell as a negative regulator of APE1/Ref-1 expression. Moreover, chromatin immunoprecipitation assays revealed that endogenous p53 is bound to the APE1/Ref-1 promoter region that includes a specificity protein (Sp1)-binding site, as well as p53-responsive *cis*-element. Recruitment of p53 to the APE1 promoter is induced by cellular stress and its association with the endogenous APE1/Ref-1 promoter interferes with the binding of Sp1 to the APE1/Ref-1 promoter, providing a mechanism for the downregulation of APE1/Ref-1. They proposed that repression of APE1/Ref-1 by p53 could provide a pathway for p53-dependent induction of apoptosis, in response to DNA damage, in accord with p53 functioning as a tumor suppressor (381). Busso *et al.* have recently reported that APE1/Ref-1 is also modified by ubiquitination, mediated by Mdm2.

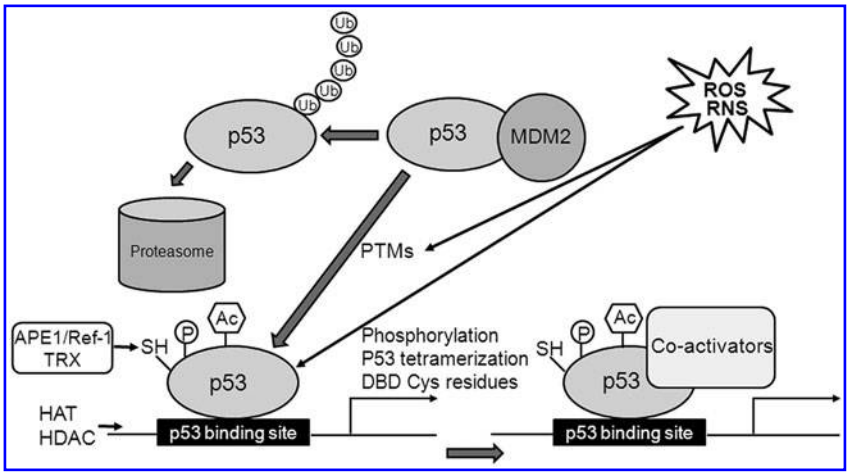

FIG. 12. Redox effect on p53 activity. A scheme demonstrating the stabilization/activation and transactivation activity of p53 is described in Figure 11. ROS and RNS affect p53 stabilization and its transactivation activity through the regulation of p53 phosphorylation. p53 phosphorylation is mediated through the action of several redox-sensitive protein kinases, including the MAPKs ERK, JNK, and p38α (321), and ATM (180), activating p53 in response to various stress signals, including ROS and RNS. p53 contains several reactive cysteine residues within its DNA-binding domain (117). Oxidation inhibits the binding of p53 to its specific binding sequence, but has no effect on its nonspecific DNA binding (272). Cys-176, Cys-238, Cys-242, and His-179 coordinate the binding of a zinc ion, which stabilizes the loop/helical structure of the core domain (59). S-glutathionylation of either Cys-124 or Cys-141, which were located by molecular modeling to the dimer interface, inhibits p53–DNA binding and interferes with the protein dimerization (322, 351). Oxidation of Cys-277 could induce conformational changes at the DNA-binding surface (117). Changes in the redox state of Cys-277 affect p53 discrimination between individual responsive elements according to their sequence (43). TRX and APE/Ref-1 significantly potentiate p53 binding to DNA by reduction of cysteine residues in the DBD. p53, in turn, regulates APE1/Ref-1 expression after genotoxic stress (381). ROS can also inactivate the HDAC activity, shifting the balance of HAT/HDAC and promoting the accessibility to p53 response element in the DNA and its transactivation activity. PTMs represent posttranslational modifications: P, Ac, and SH represent phosphorylations, acetylations, and thiols reduction, respectively.

DNA-damaging reagents increased APE1/Ref-1 ubiquitination in the presence of p53. Down modulation of Mdm2 increased APE1/Ref-1 level, suggesting that Mdm2-mediated ubiquitination can be a signal for APE1/Ref-1 degradation. These results reveal a novel regulation of APE1/Ref-1 through ubiquitination and provide further evidence for the interaction between base excision repair of DNA and the p53 signaling pathways (42).

d. The role of NO in p53 regulation. Nitric oxide is a potent activator of the p53. Data accumulated provided insights into specific mechanisms and nature of NO action under various biological contexts, suggesting that NO-induced responses are highly dependent on the levels of NO. p53 is phosphorylated and acetylated at high levels (>400 nM) of NO. It was proposed that the general tendency is that relatively low concentrations of NO tend to favor the induction of pathways, signaling for progrowth and antiapoptotic responses, whereas higher levels of NO favor pathways that induce cell-cycle arrest, senescence, or apoptosis. When NO level passes the required threshold (>400 nM) and is sufficiently high to induce p53 phosphorylation, it triggers cytostatic or apoptotic response [for review see Ref. (332)]. Early studies have revealed that induction of iNOS or exposure to exogenous NO results in p53 accumulation and the activation of p53-dependent transcription. RNS-induced $NOS^{-/-}$ macrophages were found to be defective in nuclear p53 localization, following the induction of DNA damage. Experiments in cells that are p53 null or carrying a mutant p53 gene revealed that these cells were less vulnerable to RNS, in accord with the notion that p53 may transmit a proapoptotic RNS response. It has been shown that induction of p53 by NO is preceded by a rapid decrease in Mdm2 protein, which may enable the initial rise in p53 levels early after exposure to NO. It has been further shown that NO induces p53 nuclear retention by inhibiting the Mdm2-mediated p53 nuclear export [recently reviewed in Refs. (37, 122, 188, 332, 392)].

In summary, the interrelation between ROS and p53 plays a major role in cell metabolism and survival. p53 modulates the intracellular levels of ROS, by regulating expression of both pro and antioxidant activities, as well as by regulating energy metabolism and thereby the generation of ROS, through the control of mitochondrial respiration, glycolysis, and the phosphate pentose shunt. ROS, in turn, affect the regulation of p53. Indirect effect of ROS on p53 regulation is through the modulation of both upstream signals, which regulate the triggering of p53 activity, and downstream factors, affecting its interactions with other regulatory pathways and its tumor suppressor function. ROS also affects the function of p53 by the direct interaction with critical cysteine thiols, which results in the modulation of the protein structure, affecting both its ability to bind DNA and the selectivity of binding to promoters of its target genes. S-glutathionylation of thiol residues, residing at the dimer interface (Cys-124 and 141), interferes with the protein dimerization. Oxidation of reactive cysteine residues at the core DBD (Cys-124, 141 and 182) inhibits p53 binding to DNA. Remarkably, exposure of purified p53 to H_2O_2 inhibits the binding of the protein to its specific binding sites, but has no effect on its nonspecific binding to DNA. Recent studies revealed that the redox state of Cys-277, which has been shown to induce a conformational change at the DNA-binding surface, also affects the capacity of p53 to discriminate between responsive elements, depending on their individual sequences. Of particular interest are the interrelations of p53 with APE1/Ref-1, which reduce critical cysteine residues in p53, while its expression is regulated by p53, through p53 specific response element in its promoter region. Finally, data accumulated indicate that induction of p53 by NO is concentration dependent, revealing a general tendency that at low concentrations of NO the induction of

pathways signaling for antiapoptotic responses is favored, whereas at its higher concentrations, pathways that signal for cell cycle arrest or apoptosis are induced.

F. Common characteristics in the redox regulation of transcription factors

The mammalian transcription regulation systems described here demonstrate the nature of redox signaling in metazoa, in which ROS and RNS signals are generated by intracellular processes and are used in regulatory mechanisms. Recent studies of these control mechanisms have revealed that redox regulation of protein–DNA interactions is more complex than was earlier anticipated. The complexity stems from the fact that the final event, in which a specific *trans*-acting regulatory protein binds to its *cis*-responsive sequence element in the genome, is controlled not only through the direct interactions of ROS and RNS with reactive cysteine residues in its DNA-binding site. Instead, it involves several tiers of regulatory mechanisms, including the control of protein expression, its activation, stabilization, translocation into the nucleus, oligomerization and interaction with coactivators, targeted remodeling of the chromatin structure, and, finally, the specific protein–DNA interactions with a responsive element in the genome. These regulatory mechanisms are demonstrated here in a selected group of transcription factors.

The intracellular level of transcription factors reflects the balance between the rates of their synthesis and their controlled targeted degradation in the proteasome. Both processes have been found to be affected by ROS/RNS. Several variations of a common strategy, which is based on the tight regulation of protein degradation, have been developed for the regulation of the level of transcription factors under normal cell growth and in response to specific stimuli. A common basic mechanism shared by several transcription factors is the redox-sensitive association of the transcription factor, before its activation by physiological stimuli, with an inhibitory protein that sequesters the activity of the transcription factor, impairs its nuclear translocation, and destabilizes it. An example of this strategy is demonstrated in the regulation of HIF (145, 297). The HIF-α protein is constitutively expressed in the cell, but its level in normoxia is kept low by its continuous degradation. HIF-α degradation is mediated via hydroxylation of conserved proline residues, by specific prolyl hydroxylases of the PHD family, whose activity is dependent on oxygen. Hydroxylation generates a binding site for the pVHL protein, a ubiquitin ligase recognition protein, leading to HIF-α ubiquitination and targeting for proteasomal degradation. Hypoxia suppresses the rate of HIF hydroxylation and thereby impedes the proteasomal degradation of HIF-α, resulting in the accumulation of HIF-α. ROS impede HIF-α hydroxylation by interfering with the reduction of the Fe^{3+} generated during the reaction to Fe^{2+}, which is required for the hydroxylation reaction, and thereby impairs the hydroxylation of HIF-α, leading to its stabilization (280). This pattern of regulation through the coupling of a transcription factor with an inhibitory protein, which promotes its degradation, or impairs its activity, is also represented in the case of other transcription factors, such as Nrf-2, NF-κB, AP-1 (through the regulation of JNK), and p53. Nrf2 activity in the cytoplasm is sequestered by its complex with its Keap1 inhibitor (133). Keap1 functions as a substrate adaptor for a cullin-dependent E3 ubiquitin ligase complex (69, 174), targeting Nrf2 for ubiquitination and subsequent proteasomal degradation (227, 376, 385). Stimulation by ROS or RNS results in the accumulation of free Nrf-2 (fNrf-2), either as a result of oxidation of Keap1 and the dissociation of its complex with Nrf2, following the classical hinge and latch model, or, alternatively, as the result of interference with the generation of Keap1–Nrf-2 complexes, as has been recently suggested (192). The consequence is that the free Nrf-2, which escaped ubiquitination and proteasomal degradation (87, 175, 385), can now translocate into the nucleus. The case of p53 demonstrates another example of this regulatory pattern, in which the level of the transcription factor is controlled by regulating its stability. Under normal growth conditions, the level of p53 protein is low (16, 178, 336), as it is bound by its Mdm2 inhibitor. Mdm2 blocks the interactions of p53 with transcriptional coactivators and ubiquitinates it, targeting it for proteasomal degradation (66, 319). Stress-related signals activate and stabilize p53, primarily through posttranslational modifications. ROS mediates the regulation of p53 stabilization through its effect on p53 interacting partners, including Mdm2 (122), and by affecting p53 stabilization through its phosphorylation by redox-sensitive protein kinases, such as the MAPK ERK, JNK, and p38α (321), and by ATM (180). Another variation of this protein activation–stabilization regulation strategy is demonstrated in the case of NF-κB. In the cell cytoplasm, NF-κB is bound to its IκB inhibitor and it is inactive, as its NLS site is masked by the bound inhibitor (120, 274). Cytoplasmic NF-κB is activated via several distinct cellular pathways. In the canonical pathway (120, 274), in response to a stimulus, an IκB kinase (IKK) phosphorylates IκBα, leading to its ubiquitination and its degradation by the 26S proteasome (120), releasing free NF-κB that can then translocate into the nucleus. As discussed above, although the role of ROS in the regulation of NF-κB activation has been debatable, a series of reports support the involvement of ROS in the regulation of NF-κB activation, through the action of redox-sensitive protein kinases (115, 267), which function in the dissociation of the IκB–NF-κB complex (144, 267, 341). Yet, an additional variation of the mechanism of redox-sensitive association with a negative regulator is demonstrated in the case of AP-1 activation, through the control of its upstream activating pathway. MAPKs are involved in the activation of AP-1. JNK and p38 are activated by the redox-sensitive ASK-1. It has been found that GST is involved in the regulation of both JNK-1 and ASK-1 (340). GST–ASK1 association suppresses ASK1 activity under nonstressed redox conditions. ROS induce the dissociation of the GST–ASK1 complex, resulting in the activation of Ask-1 (7, 340). It has also been found that under nonstressed conditions GST–JNK association limited the phosphorylation of Jun. Exposure to ROS resulted in the dissociation of the GST–JNK complex and enabled the phosphorylation of Jun (7). ASK-1 kinase activity is also blocked by its association with TRX in nonstressed cells. In the presence of ROS, upon oxidation of TRX, the ASK-1–TRX complex dissociates, resulting in ASK-1 activation (293).

Upon their activation in the cytoplasm, transcription factors are accumulated in the nucleus. A common feature shared by many of these proteins is that their binding to DNA and the resulting transactivation of their target genes requires their oligomerization, mostly their homodimerization or heterodimerization. The composition of the dimers is often

significant to the particular physiological functions of the individual transcription factor in the distinct pathways that it controls. Active AP-1 is bound to the DNA as a dimer consisting of various combinations of homodimers and heterodimers of the AP-1 protein family. Similarly, NF-κB forms homodimers or heterodimers with the various NF-κB proteins, and p53 is active as a tetramer. Unlike these proteins, HIF and Nrf2 are each active only as heterodimers. HIF heterodimer consists of inducible HIF-α and a stable HIF-β, and that of Nrf2 consists of Nrf-2 and Maf. ROS/RNS may affect the proteins dimerization/oligomerization, as has been found in the case of p53, where S-glutathionylation of thiol residues residing at the dimer interface interferes with the protein oligomerization (322, 351). As discussed earlier, interactions of the homodimers or heterodimers of transcription factors with their DNA recognition sites (discussed below) are promoted by the local chromatin remodeling and are followed by recruitment of transcriptional coactivators, linking the transcription factor to the transcription machinery and thereby enabling the initiation of transcription of target genes.

The transcription factors discussed here contain reactive cysteine residues in their DNA-binding domains. These cysteine residues serve as targets for ROS and RNS, which oxidize their reactive thiol groups and thereby modulate the conformation of the DNA-binding domain and inhibit their binding to the DNA, resulting in the inhibition of their transcription activity. Hence, the overall effect of ROS and RNS on the function of transcription factors presents an apparent paradoxical phenomenon, as they promote the activation of transcription factors in the cytoplasm, but exert an inhibitory effect on their function as transactivators in the nucleus (144). Another related question is how an oxidation signal affects a thiol group in a nuclear protein, considering the reductive environment in the nucleus. Our current concepts of the factors that may affect the specificity of redox signaling could provide some leads for a better understanding of these issues.

An important component, affecting the redox state of nuclear proteins, is the action in concert of APE1/Ref-1 and TRX, as well as the redox chaperon activity of APE1/Ref-1, which efficiently reduces critical cysteine thiols in the DNA-binding domains of transcription factors, enabling their binding to their specific responsive elements in the genome (12). Although it is currently accepted that the nucleus generally provides a reductive environment, through its contents of the redox pairs GSH/GSSG, NADH/NAD^+, and NADPH/$NADP^+$, a recent report demonstrates that nuclear levels of GSH/GSSG fluctuate significantly with the progress in the cell cycle, in a periodical manner (221). This could apparently result in periodical fluctuations in the nuclear reductive capacity and, consequently, may affect the capacity of ROS and RNS to affect targeted nuclear proteins during different phases of the cell cycle. In addition, as discussed earlier in this review, factors affecting the specificity of redox signaling may play a major role in directing a signal mediated by ROS or RNS to its specific target protein in either the nuclear or the cytoplasmic cellular compartments. Emphasis is made here on the potential functional role of redox sensors in the specific transmission of a redox signal. It has been suggested that transmission of the redox signal to a specific thiol group in a target protein may be mediated through the interaction of a sensor protein that sense and transmit the signal, rather than by the direct oxidation of the target protein by ROS. Further experimental work that will elucidate the routes used in transduction of the redox signal in mammalian cells will shed light on these basic questions, which are pertinent to the understanding of the specificity in redox signaling.

III. Redox Regulation of DNA Replication

Replication of the genome is a fundamental process in the life of all organisms. Its regulation is the subject for cellular control mechanisms, in which multiple proteins act in concert to ensure the accurate schedule and the fidelity of duplication of the genetic information. The processes underlying the events at the onset of DNA replication were highly conserved during evolution and their principles were defined almost five decades ago in the replicon model (134). This model proposed that sequence elements, the replicators or replication origins, determined genetically the sites in which DNA replication initiates. Initiation of DNA replication at these sites occurs through their interaction with *trans*-acting regulatory factors, the initiators, knows also as origin-binding proteins or origin recognition complexes, which link the process of replication initiation with cell growth and division. Signals transmitted by cellular control mechanisms trigger the process of DNA replication initiation at the replication origins. The genomes of prokaryotes usually consist of a single replication origin, constituting a single replication unit (replicon), whereas the nuclear genome of eukaryotes consists of multiple such sites, generating a multireplicons structure [recently reviewed in Refs. (8, 82, 118, 287, 296, 324, 368)].

The interaction of replication initiation proteins with replication origin sites is a major target of cell-cycle signaling pathways, which mediate the main cellular control of genome replication. While the role of protein phosphorylation in the control of these interactions has been studied in many replication systems, the role of redox signaling in the control of DNA replication has yet to be established. The functional role played by redox signaling in the regulation of eukaryotic cell cycle has been reported in several studies and has been the subject of a recent review (231). Here, I discuss several reports that document the role of redox signaling in the regulation of specific protein–DNA interactions during the initiation of DNA replication, as well as during DNA synthesis, focusing on the redox regulation of two replication proteins, the replication protein A (RPA) and the trypanosomal kinetoplast DNA origin-binding protein, known as the universal minicircle sequence (UMS)-binding protein (UMSBP).

A. Replication protein A

1. RPA structure and function. RPA [recently reviewed in Refs. (92, 294, 395)] is a heterotrimeric single-stranded DNA (ssDNA)-binding protein that consists of three subunits, RPA70, RPA32, and RPA14 (Fig. 13). It was originally identified as a protein required for replication of simian virus 40 (SV40) DNA *in vitro*. RPA functions in the activation of the eukaryotic prereplication complex to form the initiation complex, as well as for the loading of DNA polymerase α-primase complex onto the replication origins. It binds to stretches of ssDNA, generated during DNA replication and repair, with a $5' \rightarrow 3'$ polarity, and stimulates the action of DNA polymerases α, δ, ε, λ, and κ, during strand elongation in the processes of DNA replication and repair (Fig. 14) (210, 294, 370).

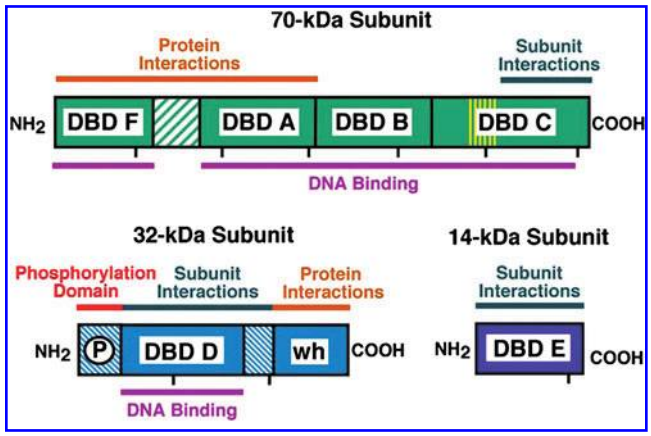

FIG. 13. Structural and functional domains of RPA. The DBDs are labeled. Regions interacting with proteins and/or DNA are indicated by horizontal lines. The conserved zinc finger motif is indicated by vertical lines. The hash marks denote 100 amino acid increments. DBD, DNA binding domain; wh, winged helix domain. Reprinted with permission from Elsevier (24), Copyright (2004). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

RPA is ubiquitous and is essential in eukaryotic cells, not only for chromosomal DNA replication but also in DNA repair, recombination, and damage signaling (210, 309, 370, 394). It functions in the initiation of homologous recombination through its interactions with Rad51 and Rad52 and was also implicated in meiotic recombination. It was found to function at sites of DNA damage, interacting with the xeroderma pigmentosum group A (XPA), stimulating its DNA binding, and recruiting the incision proteins excision repair cross-complementing 1 (ERCC1)/XPF and XPG to the damaged site. RPA interacts with many other proteins involved in DNA replication, recombination, and repair [reviewed in Ref. (294)]. Mutational analysis of the RPA70 subunits in yeast revealed the function of RPA as a checkpoint protein (205). It was found that RPA interacts specifically with the tumor suppressor p53 (85, 237) and promotes DNA binding of ATR *in vitro* (394). Recently, RPA has been identified as a regulator of mitotic transit under genotoxic stress conditions (9, 10).

RPA is a heterotrimer that consists of six OB (oligonucleotide/oligosaccharide-binding)-folds, which are tethered to each other through flexible linkers, and one HTH domain. The RPA70 subunit contains four OB-folds and the RPA32 one OB-fold, which is flanked at its N-terminus by unstructured domain and at its C terminus by the HTH domain. The RPA14 subunit contains one OB-fold. The RPA70C OB-fold (Fig. 13) contains a zinc finger domain and a 3HB (three helix bundle), which have not been detected in the other OB-loops in the protein. RPA binds ssDNA using four ssDNA-binding OB-fold domains, three of which are located at the RPA70 and one at the RPA32 [reviewed in Refs. (29, 92)]. Recently, description of the structure of the full-length RPA14/32 complex has been reported (75). The N-terminus of the RPA32 subunit contains nine sites that are subject to phosphorylation. It has a disordered structure in its unphosphorylated state (75). In response to DNA damage the N-terminus of the RPA32 subunit of human RPA becomes hyper-phosphorylated, resulting

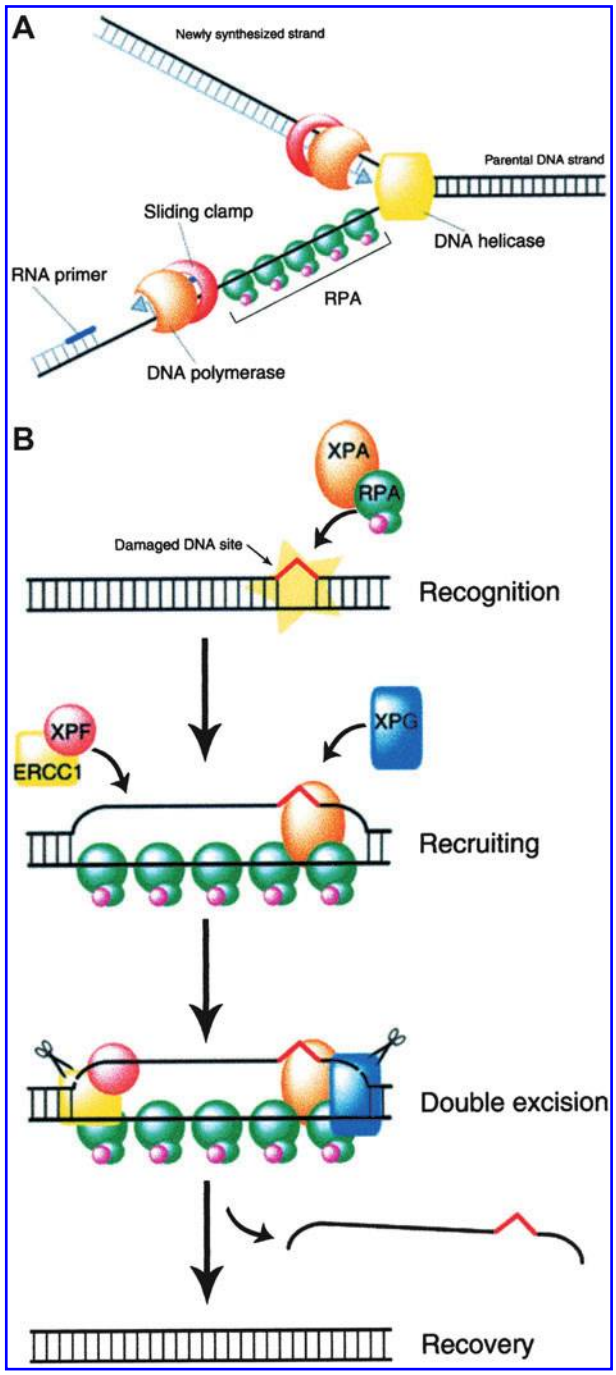

FIG. 14. The function of RPA in DNA replication and nucleotide excision repair. Function of RPA during DNA replication (**A**) and nucleotide excision repair (**B**) is illustrated. RPA accumulates along stretches of ssDNA generated during DNA replication and repair. During strand elongation in DNA replication/repair, RPA stimulates the action of DNA polymerases pol α, pol δ, pol ε, pol κ, and pol λ. RPA interacts with XPA at sites of DNA damage, stimulating XPA–DNA contact and recruiting the incision proteins ERCC1/XPF and XPG to the damaged site. NER, nucleotide excision-repair. Reprinted with permission from Wiley, Inc. (294), Copyright (2009). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

in a conformational change in RPA that downregulates its DNA replication activity, but does not affect DNA repair processes, promoting DNA repair over DNA replication activities [reviewed in Refs. (9, 24)]. Binz and Wold have recently found that the RPA70 N-terminal domain interacts with the phosphorylated domain of the RPA32 subunit and blocks its interaction with the core DNA-binding domain (25).

2. The redox regulation of RPA activities

a. The role of the RPA70 subunit zinc finger domain in redox regulation of RPA. RPA is a cysteine-rich protein containing 15 cysteine residues. Four of these cysteines, which are highly conserved, are located in the zinc finger domain of the C-terminal region of the RPA70 subunit. Earlier studies have focused on understanding the role of the RPA70 subunit zinc finger domain, *per se*, in the regulation of RPA function, its DNA-binding and unwinding activities, stimulatory effect on DNA polymerases, and participation in DNA repair mechanisms (81, 164, 183, 195, 196, 360). The function of the RPA70 zinc finger domain as the target for redox-mediated regulation of RPA activity has been documented in a study by Park *et al.*, demonstrating an order of magnitude stimulation in RPA–ssDNA interaction in the presence of the reducing agent DTT (>2 mM), and its significant inhibition by treatment of RPA with the oxidizing agent diamide (0.4–4.0 mM). On the basis of the reversibility of the redox effect on RPA's DNA-binding activity, these investigators suggested that it may regulate RPA ssDNA-binding activity. Moreover, these studies have shown that TRX was able to support the stimulation of RPA DNA-binding activity, and that a zinc finger mutant (Cys-486 to Ala substitution) was not affected by redox, suggesting that it may mediate the modulation of RPA activity *in vivo* through the effect on cysteine residues in the RPA70 zinc finger domain (271). Further analyses have revealed that mutation at any of the cysteine residues in the RPA70 zinc finger impaired the redox effect on RPA–ssDNA interactions, suggesting that all four zinc finger cysteines are required for the regulation of DNA-binding activity. Reactivity of these cysteine residues have been assessed using 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB, 50 mM). Further, these studies have shown that redox affects the initial interactions of RPA with ssDNA but has no effect on the stable RPA–DNA complex (380). A structure–function relationship study in yeast revealed that the redox regulation of RPA–ssDNA interactions is mediated through its RPA32 and RPA14 subunits, whereas formation of a stable complex with ssDNA by the RPA70 subunit *per se* is not affected (140, 162).

b. Redox affects structure and function of RPA. Using liquid chromatography/tandem MS analysis, Men *et al.* have characterized H_2O_2-oxidized RPA to identify the cysteine residues that are preferentially oxidized and to determine whether higher-level oxidation products (i.e., cysteine sulfinic acid and cysteine sulfonic acid) are induced in RPA by H_2O_2 (at the range of 2.7–10.8 mM) treatment. These analyses revealed the preferential oxidation of the four cysteine residues residing in the zinc finger motif of the RPA70 subunit, while the other 11 cysteines in this protein remained unaffected. Formation of two pairs of intramolecular disulfides (Cys-481–Cys-486 and Cys-500–Cys-503) could be generated, while no cysteine sulfinic or sulfonic acid could be found (230). Using X-ray fluorescence emission spectroscopy, Bochkareva *et al.* have found that the C-terminal domain of RPA (RPA70-CTD), containing the zinc finger motif (Fig. 13), has a coordinated Zn^{2+}, whose depletion significantly decreases the DNA-binding activity of RPA. These observations also indicated that Zn^{2+} is essential for stabilization of the tertiary structure of RPA70-CTD (30). Surface plasmon resonance (SPR) analysis of RPA–ssDNA interactions, in wild-type (wt) versus zinc finger mutant, revealed an increase of three orders of magnitude in the binding affinity of wt RPA–ssDNA interactions under reducing conditions, compared to the binding affinity measured under nonreducing conditions. No effect of redox could be detected on the ssDNA-binding affinity of the zinc finger mutant. Depletion of the divalent ion resulted in a significant decrease in binding affinity of the wt RPA interactions with ssDNA, but not in the affinity measured with the zinc finger mutant (362). Further, it was shown that redox affected RPA's interaction with damaged DNA, but not its role in the stabilization of the XPA-damaged DNA complex. Far-UV CD spectra examination of wt-RPA and a zinc finger mutant revealed that the conformational change observed in these studies in RPA upon its binding to DNA was significantly affected by redox in wt-RPA but not in the zinc finger mutant. These observations support a role for the zinc finger domain in the structural change of RPA and establish a relationship between redox regulation and the structural change that occurs in RPA upon binding to DNA.

B. The universal minicircle sequence-binding protein

1. Structure and replication of the kinetoplast DNA of trypanosomatids. Kinetoplast DNA (kDNA) is a DNA network found in the single mitochondrion of flagellated protozoa of the Kinetoplastida. Several members of this group, belonging to the Trypanosomatidae, are the agents of severe and widely spread tropical diseases, such as the trypanosomal South American Chagas disease and the African sleeping sickness, as well as the various forms of leishmaniases. Diverging early from the main lineage of eukaryotes, trypanosomatids developed distinct molecular mechanisms (314). Among these are the use of trypanothione ($T[SH]_2$)-based tryparedoxin (TXN)/tryparedoxin peroxidase (TXNPX) redox pathway, in the regulation of their intracellular redox state [recently reviewed in Ref. (177)], as well as the development of the remarkable kDNA structure. kDNA is a topological network consisting of ~5,000 duplex DNA minicircles and 25–50 maxicircles that are interlocked topologically into a DNA catenane, which is condensed into a disk-shaped structure in the mitochondrial matrix (199, 206, 312). Its replication [recently reviewed in Refs. (199, 206, 312)] includes the duplication of minicircles and maxicircles and the segregation of the network into two progeny networks upon cell division. Before their replication, covalently closed minicircles are released from the network and replicate, as individual replicons, through theta (θ) structure intermediates, which accumulate at two protein assemblies located at antipodal sites flanking the kDNA disk, where primer removal, repair, and ligation of most gaps between Okazaki fragments occur. Then, a topoisomerase II reattaches the progeny minicircles onto the network, where final gap filling and sealing occur.

2. Structure and function of the kDNA minicircle origin-binding protein UMSBP. Two sequence blocks in the mini-

circle molecule were conserved in all trypanosomatid species studied, a 12-mer (GGGGTTGGTGTA), known as the universal minicircle sequence (UMS), and a hexamer sequence (ACGCCC), which were proposed as the replication origins of the minicircle's DNA strands. The conserved UMS and the hexameric sequence (the latter in the context of flanking origin sequences) are bound specifically by UMSBP (3, 4, 343–345).

The UMS binding protein (UMSBP) was first purified from the trypanosomatid species *Crithidia fasciculata*, as a homodimer of 27.4 kDa with a 13.7-kDa protomer (344). It is a sequence-specific single-stranded DNA-binding protein that interacts specifically with the two conserved minicircle origin sequences (4, 343). The gene encoding UMSBP in *C. fasciculata* consists of an ORF, encoding a polypeptide chain of 116 amino acid residues that contains five CCHC-type zinc finger motifs (3, 345) (Fig. 15). Structure–function analysis, using site-directed mutagenesis, revealed that the five CCHC-type zinc finger motifs predicted in UMSBP fold into zinc-dependent structures, capable of binding a single-stranded nucleic acid ligand. The five zinc fingers in UMSBP differ in their function. While the zinc fingers located at the protein's C-terminal domain are involved in DNA binding, the N-terminal zinc finger is involved in protein–protein interactions that lead to UMSBP oligomerization. UMSBP binds the conserved origin sequence as a monomer, in a zinc-dependent manner (260). Orthologs of the *UMSBP* gene have been found in the genomes of all other trypanosomatids analyzed (238).

The function of UMSBP *in vivo* has been studied in *Trypanosoma brucei*, using RNA interference (RNAi) analysis (238). Simultaneous silencing of the two *T. brucei UMSBP* genes resulted in remarkable effects on the trypanosome cell cycle. It significantly inhibited the initiation of minicircle replication, confirming the function of UMSBP *in vivo* as an initiator protein during the replication of kDNA minicircles. Remarkably, these analyses also revealed that silencing of the *UMSBP* genes impaired the segregation of kDNA networks and the flagellar basal body, and impeded nuclear division, leading to cell growth arrest. These observations revealed the function of UMSBP in postreplication activities and implied its potential role in linking kDNA replication and segregation to the nuclear S-phase control, during the trypanosome cell cycle.

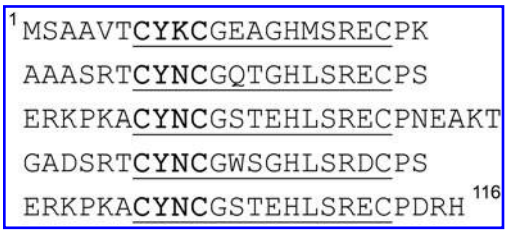

FIG. 15. Zinc fingers and Cys-X-X-Cys domains in UMSBP. The 116-amino-acid monomer consists of five CCHC-type zinc finger domains (*underlined*). The N-terminal zinc finger is involved in UMSBP oligomerization, while the C-terminal zinc fingers are involved in the binding of UMS DNA. The five Cys-X-X-Cys motifs within the zinc fingers are indicated (*boldface*). This research was originally published in the Journal of Biological Chemistry: Sela D, Yaffe N, and Shlomai J. 2008. Enzymatic mechanism controls redox-mediated protein–DNA interactions at the replication origin of kinetoplast DNA minicircles. 283: 32034–32044. Copyright, the American Society for Biochemistry and Molecular Biology, Ref. (305).

3. Redox regulation of UMSBP

a. Redox affects the binding of UMSBP to the replication origin and its oligomeric state. UMSBP contains 15 cysteine residues located in five CCHC-type zinc finger domains (Fig. 15). Atomic absorption spectroscopy revealed the intrinsic association of zinc ions with UMSBP (260) (Onn *et al.*, unpublished). UMSBP–DNA interactions are zinc dependent. Pretreatment of UMSBP with 1,10-*o*-phenanthroline (but not the nonchelator 1,7-phenanthroline) significantly inhibited its capacity to bind UMS DNA in an EMSA analysis, whereas addition of zinc ions partially restored the protein capacity to interact with the UMS DNA ligand. Zinc depletion was found to affect the structure of UMSBP, as shown by gel filtration analysis (260).

The effect of redox on the DNA-binding activity of UMSBP was examined by monitoring the effect of either a reducing agent (DTT, 0.02–20 mM) or an oxidizing agent (diamide, 0.01–1.0 mM; H_2O_2, 20 mM) on the binding of UMSBP to UMS DNA, in EMSA analysis. In the absence of DTT, almost no DNA-binding activity of UMSBP could be detected. Increasing the concentration of DTT resulted in a concomitant increase in the generation of protein–DNA complexes, whereas increasing the concentration of the oxidizing agent resulted in a concomitant decrease in the generation of UMSBP–UMS nucleoprotein complexes. Inhibition of the DNA-binding reaction by *N*-ethyl maleimide confirmed that the effect of redox on the DNA-binding activity of UMSBP is mediated through its zinc fingers' thiol groups. The reversible nature of redox effect on UMSBP activity raised the possibility that UMSBP–UMS DNA interactions could be mediate by redox (260).

The reduced UMSBP monomer is the only form of the protein that binds DNA. UMSBP oligomerizes in the presence of thiol-oxidizing agents and reversibly monomerizes in the presence of a thiol-reducing agent (260). Hence, it has been suggested that UMSBP binding to DNA may be regulated, via the redox-mediated reversible interconversions of active UMSBP monomers and its inactive oligomers (260). This model has been challenged experimentally, by correlating the DNA-binding capacity of UMSBP and its oligomerization, in the presence of increasing concentrations of the oxidizing agent diamide (0.1–1.0 mM). No correlation could be observed in these experiments between the inhibition of UMSBP DNA-binding activity and its oligomerization. On the basis of these observations it was suggested that UMSBP inactivation, in the absence of oligomerization, could rather be the result of the generation of intramolecular disulfide bonds by the oxidized thiols in the zinc fingers' cysteine residues. Moreover, it was found that while the presence of zinc ions was essential for the interaction of UMSBP with DNA, UMSBP oligomerization occurred through zinc-depleted, unfolded zinc finger domains (304). The correlation between UMSBP DNA-binding activity and its oligomeric state was examined *in vivo* in *C. fasciculata* cells exposed to increasing concentrations of H_2O_2. In accord with the *in vitro* observations, the results revealed that while DNA-binding activity was significantly impaired in the presence of the oxidizing agent (at the range of 0.01–10 mM H_2O_2), no detectable change could be observed in UMSBP's oligomeric state *in vivo* under these conditions (304).

Overall, these results implied that the redox regulation of UMSBP interactions with DNA is not mediated through redox effect on the protein oligomeric state.

SPR analysis was used to determine whether redox affects the association of the free unbound UMSBP with DNA or its dissociation from the nucleoprotein complex. This question is pertinent to the understanding of redox regulation of UMSBP binding to the replication origin and the consequent generation of a replication initiation complex. Equilibrium binding constants and kinetic parameters, measured under either reducing or oxidizing conditions during the association and/or dissociation stages of the reaction, revealed that redox affects the association of UMSBP with DNA, but has little effect on the dissociation of prebound UMSBP from the nucleoprotein complex (304).

b. Regulation of UMSBP through the cell cycle control of its redox state. Studies conducted in synchronized *C. fasciculata* cell cultures have shown that the steady-state levels of UMSBP mRNA and protein are apparently constant throughout the entire trypanosomatid cell cycle (5), suggesting that regulation of UMSBP activity *in vivo* is most probably of a posttranslational nature. This presumption is in accord with the possibility of redox regulation of UMSBP activity *in vivo*. This question was addressed in synchronized cell cultures, monitoring both UMSBP's DNA-binding activity and its redox state, throughout the trypanosomatid cell cycle (305). These analyses revealed that UMSBP activity fluctuates in a cell-cycle-dependent manner, displaying two peaks of UMS DNA-binding activity, one during S phase and the other during M-G1. Cycling of UMSBP activity was suppressed when measured under reducing assay conditions, suggesting that cycling of UMSBP activity reflects the cycling of its redox state during the progress of the cell cycle. The periodic fluctuation of UMSBP's redox state was also confirmed by analysis (214) of its redox state during the cell cycle, revealing that cycling of UMSBP's redox state correlates with its UMS-binding activity. These observations suggested that UMSBP activity is regulated *in vivo* through the cell-cycle-dependent control of the protein's redox state (305). The two distinct peaks of UMSBP activity observed during the cell cycle are in accord with its S phase function as an initiator protein during kDNA replication, and its recently reported postreplication activities in the segregation of kDNA and the basal bodies and cytokinesis (238).

c. Potential function of the TXN/TXNPX redox pathway in the regulation of UMSBP. The fluctuations of UMSBP redox state *in vivo*, displaying relatively higher levels of reduction during S and M-G1 phases and higher levels of oxidation during other stages of the cell cycle, implied that both reduction and oxidation of UMSBP are tightly regulated during the progression of the trypanosomatid cell cycle. The possibility that redox regulation of UMSBP is mediated by an enzyme-based mechanism has been challenged by coupling *in vitro* the UMSBP–UMS DNA-binding reaction to the main metabolic pathway that regulates the intracellular redox state in trypanosomatids. As mentioned above, this pathway differs in trypanosomatids, by several features, from redox regulating pathways found in other eukaryotic systems (Fig. 16). While most eukaryotes use the GSH/GR and the TRX/TRXR systems for maintaining their intracellular thiol redox homeostasis, trypanosomatids lack the genes encoding GR and TRXR. Instead, their redox metabolism is based on trypanothione (TS_2) and trypanothione reductase (TR), functioning in a redox cascade pathway with NADPH as the primary source of electrons, where TR reduces TS_2 to yield $T[SH]_2$, which is capable of reducing TXN, which in turn reduces the 2-Cys PRX, TXNPX, as well as the GSH peroxidase-type TXNPX, GPX. These peroxidases can then reduce and detoxify hydroperoxides in the cell [reviewed in Refs. (89, 177)]. Several earlier observations supported the notion that enzymes of the trypanothione-based redox pathway, TXN and TXNPX, are likely candidates to play a role in the regulation of UMSBP's redox state. First, TXNPX and TXN were found to interact with UMSBP by both yeast two hybrid analyses and (TXNPX) by affinity chromatography of cell extracts on UMSBP attached to solid phase, followed by MS analysis (305) (Milman and Shlomai, unpublished). It has also been reported that overexpression of *T. brucei* TXNPX resulted in an increase in UMSBP oligomerization and loss of kDNA (244). Finally, UMSBP contains five 2-Cys motifs (Cys-X-X-Cys), one within each of its zinc fingers (Fig. 15), rendering UMSBP a potential substrate for oxidation by the 2-Cys peroxyredoxin TXNPX (95).

A UMSBP–UMS-binding reaction consisting of preoxidized UMSBP and ^{32}P-labeled UMS DNA was coupled to a reaction reconstituted from pure trypanosomal proteins that contained NADPH and TS_2, TR, and TXN. EMSA analysis of the products of the coupled reactions revealed the activation of the preoxidized, otherwise inert UMSBP, resulting in the generation of UMSBP–UMS nucleoprotein complexes (260). On the other hand, coupling of a UMSBP–UMS DNA-binding reaction consisting of prereduced UMSBP and ^{32}P-labeled UMS with a TXNPX oxidation reaction consisting of TXNPX and cumene hydroperoxide, significantly suppressed the

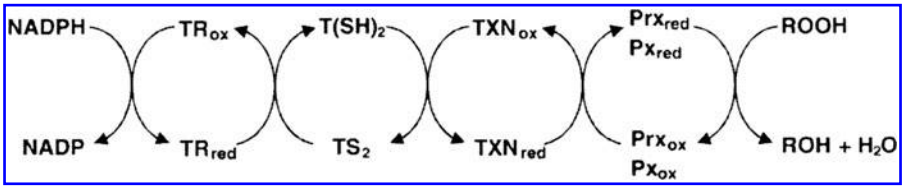

FIG. 16. The tryanothione-mediated hydroperoxide metabolism of trypanosomatids. Detoxification of hydroperoxides (ROOH) is accomplished by the 2-Cys Prx, TXNPX and GPX, both deriving their reducing equivalents from a cascade composed of TXN, trypanothione [$T(SH)_2$], and TR with NADPH as the primary electron source. It consists of the reduction of $T(S)_2$ to $T(SH)_2$ by TR, followed by the reduction of TXN by trypanothione. TXN then reduces the peroxyredoxin TXNPX and the GPX, which can, in turn, reduce hydroperoxides. The subscripts *red* and *ox* refer to the redox state of the proteins. GPX, glutathione-peroxidase (Px)-type TXNPX; Prx, peroxiredoxin; TR, trypanothione reductase; TXN, tryparedoxin; TXNPX, tryparedoxin peroxidase. Reprinted with permission from Elsevier (177), Copyright (2008).

generation of UMSBP–UMS DNA complexes (305). Coupling together the three reactions (TXN–UMSBP–TXNPX) (Fig. 17) demonstrated that this pathway, reconstituted *in vitro*, has the capacity to reversibly activate and suppress the binding of UMSBP to the origin sequence. It has been demonstrated that in the course of this reaction TXNPX directly oxidizes UMSBP, whereas TXNPX itself is being reduced (305). These observations demonstrated the capacity of TXN and TXNPX to reciprocally control the interaction of UMSBP with the origin sequence *in vitro* through their opposing effects on the redox state of UMSBP. The fact that both up- and downregulation of UMSBP activity is displayed in the reconstituted reaction suggests a potential physiological role *in vivo* of this enzymatic pathway in regulating the binding of UMSBP onto the minicircle replication origin, functioning as a redox-mediated molecular switch.

IV. Concluding Remarks

During their evolution, organisms have developed the ability not only to sense ROS and RNS and to protect their cellular components against the damage caused by these reactive molecules, but also to harness ROS and RNS for the regulation of major cellular processes. Data accumulated during the last four decades have established that redox signaling plays a significant role in the mechanisms that control the intricate networks that regulate gene expression, in response to external as well as intracellular physiological stimuli. This review has discussed a selected group of well-studied eukaryotic master transcriptional regulators that control the induction and suppression of multiple metabolic pathways, functioning in cell growth, development, differentiation, and survival. It focuses on the role played by redox in the regulation

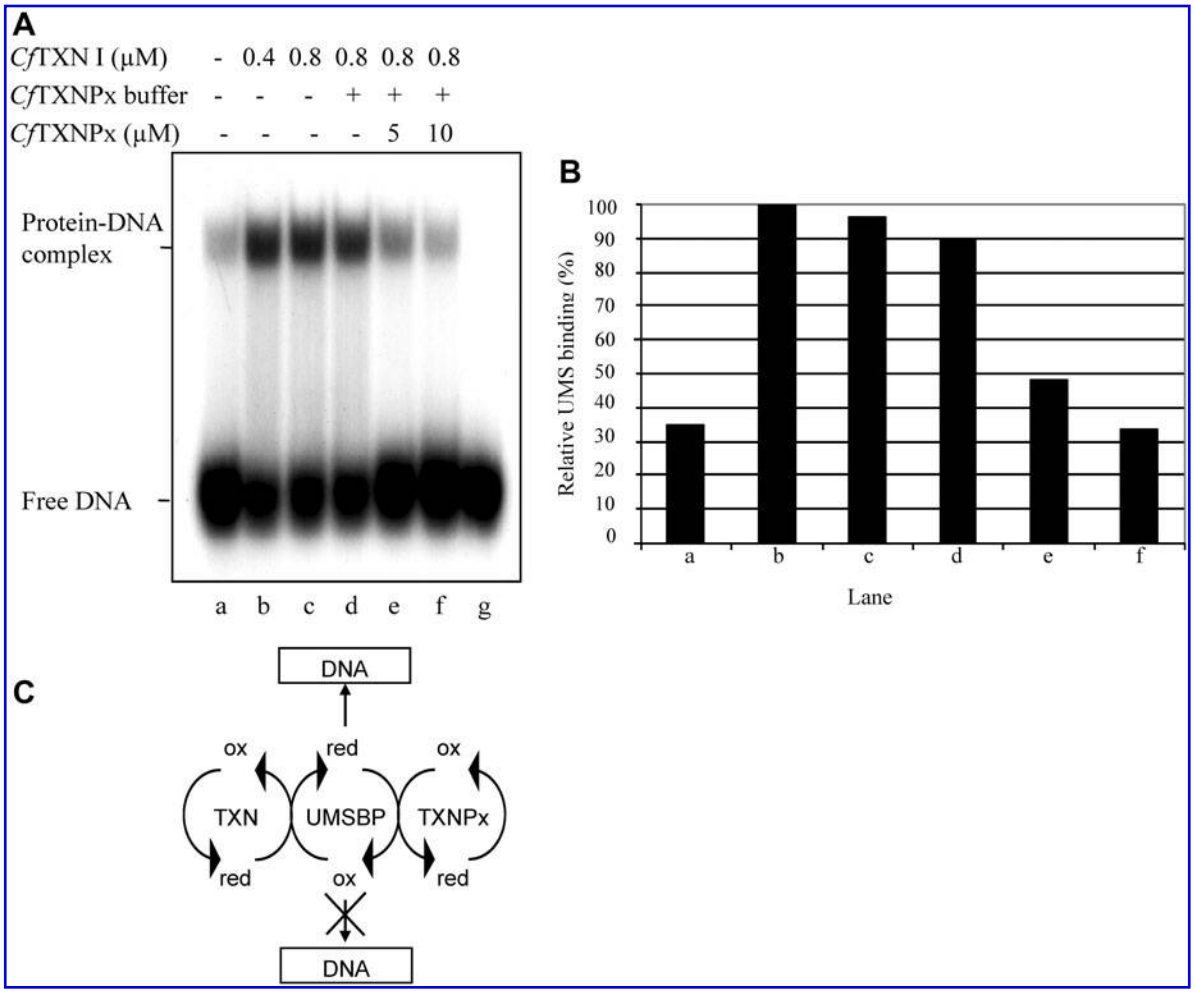

FIG. 17. Reconstituted TXN–TXNPX reaction regulates UMSBP binding to the conserved origin sequence *in vitro*. (**A**) Diamide-preoxidized UMSBP (*a–f*) was incubated in a tryparedoxin reaction (*a–g*). The reaction was followed by the addition of either the TXNPX reaction components alone (*d*) or with the indicated concentrations of TXNPX (*e* and *f*). The reactions were followed by EMSA, and quantified by phosphorimager. In *a*, no TXN was added; in *g*, no protein was added. (**B**) Phosphorimaging quantification of the EMSA data. (**C**) A proposed model for the redox-regulated binding of UMSBP to the replication origin through the opposing effects of TXN and TXNPX, serving as a redox-mediated molecular switch. *C. fasciculata* TXNI and TXNPX were used. This research was originally published in the *Journal of Biological Chemistry*: Sela D, Yaffe N, and Shlomai J. 2008. Enzymatic mechanism controls redox-mediated protein–DNA interactions at the replication origin of kinetoplast DNA minicircles. 283: 32034–32044. Copyright, the American Society for Biochemistry and Molecular Biology, Ref. (305).

of the specific protein–DNA interactions that control gene expression and genome replication.

The different transcription factors systems discussed in this review demonstrate an array of redox-regulated mechanisms that control their function in transactivation of their target genes. This includes the control of the intracellular levels of transcription factors that in most cases reflects a balance between their rate of synthesis and their controlled targeted degradation in the proteasome. It also includes the redox-mediated dissociation of their inhibitory complexes with protein partners functioning as their negative regulators. In addition, the introduction of a wide range of posttranslational modifications in both transcription factors and their upstream regulators is used in regulatory mechanisms. Within these various posttranslational modifications, a most significant level of regulation observed in both transcription and replication systems is provided by protein phosphorylation mediated by redox-sensitive protein kinases. Moreover, the modulation of local chromatin structure mediated by redox-sensitive HATs and HDACs is now recognized as an important mechanism promoting (or suppressing) the binding of transcription factors onto their responsive elements in the genome. Finally, the transcription and replication regulatory proteins discussed here contain reactive cysteine residues in their DNA-binding domains. ROS and RNS, as well as reducing enzymes, especially TRX and Ref-1, interact with these reactive cysteines and thereby modulate their conformation, affecting their DNA-binding activity. The molecular pathways that function in this complex array of control mechanisms, as well as the cross-talk between the various pathways during the regulation of protein–DNA interactions, will continue to challenge investigators in the field of redox regulation. This includes questions regarding the intracellular regulation of ROS and RNS generation, the control of their flux in response to different physiological stimuli, and their specificity of action.

Research on the role of redox signaling in the replication of DNA has been less extensive than in transcriptional regulation. Major questions regarding the significance of redox signaling in the process of genome replication are yet to be addressed. Study of the role played by redox signaling in cell cycle control [recently reviewed in Ref. (231)] is pertinent to our ability to address these questions. A cell-cycle-dependent fluctuation of both the redox state of an initiator protein and its capacity to bind the replication origin was discussed here, in the case of the kDNA origin-binding protein UMSBP (305). The pathways linking cell cycle control, intracellular redox state, and genome replication are yet to be addressed, as are the sources and types of reactive molecules that function in redox signaling of genome replication and the intracellular routes used for transmitting these signals.

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Author Disclosure Statement

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Abbreviations Used

AKR = aldo-keto reductase
AP = activator protein
AP-1 = activator protein-1
APE1/Ref-1 = AP endonuclease 1/redox factor 1
ARE/EpRE = antioxidant/electrophile-responsive element
ARF = alternative reading frame
ARNT1 = aryl hydrocarbon receptor nuclear translocator
ASK1 = apoptosis signal-regulating kinase 1
ATF = activating transcription factor
ATM = ataxia telangiectasia mutated kinase
ATR = ATM RAD3-related kinase
bHLH = basic helix-loop-helix
BTB = bric-a-brac, tram-track, broad complex
bZIP = basic leucine zipper
bZip = basic leucine zipper
CAD or CTAD = C-terminal transactivation domain
CK II = casein kinase II
CNC = cap "n" collar
CREB = cAMP response element-binding protein (p300/CBP)
CTD = C-terminal domain of RPA
DBD = DNA-binding domain
DETA-NO = (Z)-1-[2-aminoethyl-N-(2-ammoniothyl) amino] diazen-1-ium-1, 2-diolate
diamide = diazene dicarboxylic acid bis[*N*,*N*-dimethylamide]
DNAPK = DNA-dependent protein kinase
ERCC1 = excision repair cross-complementing 1
ERK = extracellular signal-regulated kinase
GPX = glutathione peroxidase
GR = glutathione reductase
GS = glutathione synthetase
GSH = glutathione
GSK3β/Fyn = glycogen synthase kinase-3β
GSNO = S-nitrosoglutathione
GSSG = glutathione disulfide
GST = glutathione-S-transferase
H_2O_2 = hydrogen peroxide
HAT = histone acetyl transferase
HDAC = histone deacetylase
HIF = hypoxia-inducible transcription factor
HRE = hypoxia response element
HTH = helix-turn-helix
IκB = inhibitor κB
IKK = IκB kinase
iNOS = inducible nitric oxide synthase
IRES = internal ribosome entry segments
JNK = Jun amino terminal kinase
kDNA = kinetoplast DNA
Keap1 = Kelch-like erythroid-cell-derived protein with CNC homology (ECH)-associated protein 1
LPS = lipopolysaccharide
Maf = musculoaponeurotic fibrosarcoma
MAPK = mitogen activated protein kinases
Mdm2 = mouse double-minute-2 protein
MEKK = MAPK/ERK kinase
miRNA = microRNA
MKK = MAPK kinase
MS = mass spectrometry
MSK-1 = mitogen and stress-activated kinase-1
mTOR = Mammalian target of rapamycin
NAC = *N*-acetyl-L-cysteine
NAD, NTAD = N-terminal transactivation domain
Neh = Nrf2–ECH homology
NEMO = NF-κB essential modifier
NES = nuclear export signal
NF-κB = nuclear factor kappa B
NIK = NF-κB-inducing kinase
NLS = nuclear localization signal
NOX1 = NADPH oxidase 1
NQO = NADPH quinone oxidoreductase
Nrf2 = nuclear factor E2-related factor 2
O_2^- = superoxide
OB = oligonucleotide/oligosaccharide binding
ODDD = oxygen-dependent degradation domain
ORC = origin recognition complexes
PAS = PER, ARNT, and SIM
PERK = protein kinase R–like endoplasmic reticulum kinase
PGM = phosphoglycerate mutase
PHD = prolyl hydroxylase domain
PI-3-K = phosphatidylinositiol-3-kinase
PKA = protein kinase A
PKC = protein kinase C
PRX = peroxiredoxins
PTEN = phosphatase and tensin homolog; pVHL, von Hippel–Lindau tumor suppressor protein
RHD = Rel homology domain
RNS = reactive nitrogen species
ROS = reactive oxygen species
RPA = replication protein A
RSK = ribosomal S6 kinase
SAPK = stress-activated protein kinase
SCO2 = synthesis of cytochrome *c* oxidase 2
SIRT1 = the mammalian ortholog of the yeast silencing information regulator 2 (SIR2)
SNAP = S-nitroso-*N*-acetylpenicillamine
SNO = S-nitrosothiol
SNP = sodium nitroprusside
SOD = superoxide dismutase
SPR = surface plasmon resonance
SRC-1 = steroid receptor coactivator-1
SUMO = small ubiquitin-like modifier
SWI/SNF = switch/sucrose non-fermentable
TAD = transactivation domain
TBB = 4,5,6,7-tetrabromobenzotriazole
tBHQ = tert-butylhydroquinone
TCR = T-cell receptor
TIGAR = TP53-induced glycolysis and apoptosis regulator
TLR4 = toll-like receptor 4
TR = trypanothione reductase
TRX = thioredoxin
TRXR = thioredoxin reductase
TS_2 = trypanothione
TXN = tryparedoxin
TXNPx = tryparedoxin peroxidase
UMS = universal minicircle sequence
UMSBP = UMS-binding protein
VEGF = vascular endothelial growth factor
XPA = xeroderma pigmentosum group A

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