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Forum Review

Redox Activation of p21$^{Cip1/WAF1/Sdi1}$: A Multifunctional Regulator of Cell Survival and Death

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ABSTRACT

Cell division requires the coordinated assembly of cyclins and cyclin-dependent kinases that promote cell-cycle progression through S phase and mitosis. Two families of cyclin-dependent kinase inhibitors prevent abnormal or premature proliferation by blocking cyclin kinase activity. Expression of the cyclin-dependent kinase inhibitor p21, a member of the Cip/Kip family, increases when cells are damaged. In addition to controlling cell-cycle progression, p21 participates in DNA repair and apoptotic processes. The recent appreciation that p21 regulates cell survival and death implies that it is a master regulator of cell fate. This review discusses how p21 can affect the cellular response to oxidative stress. *Antioxid. Redox Signal.* 7, 108–118.

INTRODUCTION

RECENT STUDIES have led to an appreciation that reactive oxygen species (ROS) are important mediators of intracellular redox signaling (114). Under most conditions, enzymatic and nonenzymatic antioxidant molecules ensure that ROS levels are tightly regulated. Oxidative damage occurs when defense mechanisms fail or when the level of ROS overwhelms the cell's capacity to reduce oxidant species. Cells may repair damage, fixate the mutation, or die by apoptosis or necrosis. Indeed, cumulative oxidative damage over time is thought to underlie both cancer and the aging process (11). Oxidative damage may also occur when cells become stressed, such as during ischemia–reperfusion or when neutrophils are recruited to sites of tissue injury (63). ROS are capable of attacking all macromolecules, including DNA. The main oxidation products of DNA include base modifications, base loss, and strand breaks (28). These DNA lesions activate cell-cycle checkpoints, which inhibit cell cycle progression presumably to allow additional time for damage to be repaired. The observation that checkpoint failure is associated with enhanced DNA lesions and cell death enforces the concept that DNA replication/repair and apoptosis are interrelated processes (66).

In response to DNA damage, cells growth-arrest in G1 and G2 phases of the cell cycle or undergo apoptosis. It is now well established that the tumor suppressor p53 is responsible for both the G1 checkpoint and DNA damage-dependent apoptosis. p53 is a transcription factor that regulates a large number of genes involved in cell growth, DNA repair, and survival (106). In this capacity, p53 is the "guardian of the genome" because it judges whether damaged cells may undergo repair or programmed cell death. The major downstream target of p53-mediated G1 arrest is the cyclin-dependent kinase (cdk) p21 $^{Cip1/WAF1/Sdi1}$ (hereafter p21). Although it was the first cdk inhibitor to be identified, subsequent research has led to a larger appreciation that p21 may also affect apoptosis, cell differentiation, DNA repair, and senescence. If p53 is the "guardian of the genome," then p21 is the "foreman" whose job is to ensure that DNA maintenance occurs when the "guardian" is absent. Indeed, p53-independent induction of p21 occurs under conditions that oxidize and inhibit p53 transcriptional activity (97). The observation that p53-independent pathways have evolved suggests that p21 is an essential component of the cellular response to oxidative stress. The current review focuses on how p21 multitasks survival and death when cells become oxidized.

ALL OXIDANTS ARE NOT EQUAL

Understanding how cells respond to individual ROS is a major challenge for investigators today. The term ROS has

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loosely been defined to include singlet oxygen, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), and the nitrosylated molecules nitric oxide ($NO^\bullet$) and peroxynitrite ($ONOO^-$). Most ROS are produced as by-products of mitochondrial respiration. As oxygen is not genotoxic by itself, it must damage DNA indirectly through formation of one or more of these species (42). Toxicity is in direct proportion to oxygen concentration and thought to be mediated by the generation of superoxide and hydroxyl radicals that attack DNA (135). Unlike these oxidant species, singlet oxygen is very inefficient at promoting DNA strand breaks (83). ROS may also oxidize lipids, which in turn damage DNA (10, 109). It remains unclear how much of the genotoxic effects of hyperoxia are manifested by hydrogen peroxide because hydrogen peroxide produces mutagenic single strand breaks, whereas hyperoxia produces nonmutagenic single and double strand breaks (43). Additionally, the metal chelator desferrioxamine inhibits hydrogen peroxide while potentiating hyperoxia-induced genotoxicity (44). Although these studies suggest hydrogen peroxide is not directly involved in oxygen toxicity, it may still contribute when converted to the more toxic hydroxyl radical. Alternatively, intracellular production of hydrogen peroxide during hyperoxia may affect cells differently than when delivered exogenously.

ROS also contribute to the toxicity of many agents. For example, the genotoxic effects of γ-irradiation [ionizing radiation (IR)] are proportional to the level of oxygen present (21). This led Gerschman *et al.* to propose that the toxic effects of hyperoxia and radiation involved the formation of a common set of free radicals (40). However, cell lines that are resistant to hyperoxia-induced killing are not also resistant to radiation (59). Conversely, radiation-sensitive cell lines that lack one or more DNA repair enzymes are not also sensitive to hyperoxia (45). Although circumstantial, these findings suggest that radiation and hyperoxia produce different free radicals that cause unique types of DNA lesions. Consistent with this concept, the proportion of DNA single and double strand breaks produced by lymphocytes or thymocytes exposed to hydrogen peroxide, bleomycin, or radiation is different (8). Collectively, these studies exemplify the complexity by which different, seemingly analogous oxidants induce different types of DNA lesions and cellular responses. As such, the cellular response to one oxidant species cannot be extrapolated to all. This is clearly evident when considering the role of p21 in the cellular response to oxidative stress.

THE MULTIPLE FUNCTIONS OF p21

How p21 was discovered

Six independent laboratories using distinct approaches identified p21. Through its association with cdk2, p21 was identified as a cyclin-dependent kinase interacting protein (Cip1) (53), a cdk2-associated protein-20 (CAP20) (48), and a 164-amino acid protein of 21 kDa (p21) (129). It was also discovered through subtractive cloning of genes induced by overexpression of p53 (30). The identification of p21 as being a downstream target of p53 transcriptional activity clarified earlier work showing that cyclin complexes isolated from *p53*-deficient cells lacked p21 (130). p21 was also identified as a senescent cell-derived inhibitor (sdi1) based on its expression in senescent fibroblasts and its ability to inhibit DNA synthesis when overexpressed (84). The observation that overexpression of p21 in HT1080 fibrosarcoma or H1299 human lung adenocarcinoma cells leads to cell senescence confirmed its role as a promoter of the senescent phenotype. p21 has also been called melanoma differentiation antigen 6 (mda6) for its cloning from differentiating melanoma cells (58).

Additional studies revealed that p21 is normally bound to other proteins. It has been detected in a binary complex with proliferating cell nuclear antigen (PCNA) (122) and a quaternary complex containing cyclins, cdks, and PCNA (129, 133). The amino-terminus of p21 binds cyclin-cdk complexes, whereas the carboxy-terminus binds PCNA (Fig. 1) (16). Within the amino-terminal domain, amino acids 17–24 interact with cyclins and amino acids 53–59 interact with cdk2. Based on structural homology within this domain, p21 belongs to the Cip/Kip family of cdk inhibitors that includes $p21^{Cip1}$, $p27^{Kip1}$ (91, 117), $p57^{Kip2}$ (67, 74) where Kip refers to kinase inhibitory protein. The PCNA binding and nuclear localization signals are located between amino acids 140 and 160. Both the amino- and carboxy-terminal domains of p21 exert G1 growth arrest when expressed separately in cells (72, 96). Although both domains inhibit proliferation, the amino-terminal domain appears to be more active. These studies collectively suggested that p21 controls cell growth and differentiation.

p21 and the G1 restriction point

Cell-cycle progression is controlled by cdks and catalytic cyclins that phosphorylate subtrates required for exit from G1 into S phase or exit from G2 to M phase. In the absence of mitogens, cells exit from the cell cycle and arrest in G0, an early stage of G1. Mitogens promote cell division by activating Ras-dependent cyclin D1 gene transcription (Fig. 2) (1). Newly synthesized cyclin D1 associates with cdk4 and cdk6, which sequester Cip/Kip proteins and PCNA to form an active quaternary complex (65). The sequestration of p21 into cyclin D/cdk complexes titrates p21 from cyclin E/cdk2 complexes, where it has higher affinity and inhibitory activity. The active cyclin D complex translocates into the nucleus and rapidly phosphorylates retinoblastoma gene product (Rb), thereby allowing additional phosphorylations in late G1 by active cyclinE/cdk2 complexes. Active cyclin E/cdk2 also promotes destruction of p27 by phosphorylating it on threonine 187 (118, 121). The destruction of p27 switches those cells in mid-G1 from being mitogen-dependent to mitogen-independent. Hyperphosphorylated forms of Rb no longer associate

FIG. 1. Structure of p21 protein. p21 is a small 164-amino acid protein with three major binding sites for cyclins (Cyc), cdks (CDK), and PCNA. A nuclear localization signal (NLS) is located within the carboxy-terminus.

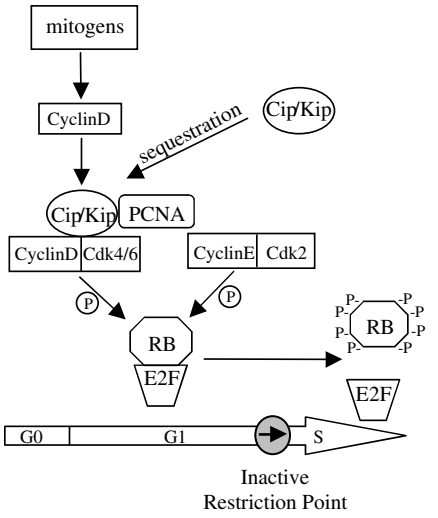

FIG. 2. Cell cycle progression from G1 into S phase. Mitogen-dependent progression through G1 involves assembly of newly synthesized cyclin D with cdk4 or cdk6, sequestration of low levels of Cip/Kip proteins, and phosphorylation (P) of the retinoblastoma gene product (Rb). Mitogen-independent assembly of cyclin E kinases occurs during late G1 and continues to phosphorylate Rb, which ultimately dissociates from the transcription factor E2F.

with the transcription factor E2F, which is free to promote transcription of genes required for S phase progression and DNA synthesis. Thus, early G1 progression involves mitogen-dependent accumulation of cyclin D, sequestration of Cip/Kip proteins, and cyclin D-dependent phosphorylation of Rb. Late G1 progression involves cyclin E/cdk2-dependent phosphorylation and destruction of p27 Kip1 and full phosphorylation of Rb. This leads to mitogen independence and bypass of the restriction point at the G1/S boundary. The observation that active cyclin D complexes fail to form in mouse embryo fibroblasts derived from mice lacking both p21 and p27 emphasizes the importance of Cip/Kip proteins to promote cell division (18).

Growth arrest involves the INK4 family of cdk inhibitors, so named for their ability to inhibit cdk4 as well as cdk6. Unlike Cip/Kip proteins that bind both G1 and S phase cyclins and cdks, the INK4 proteins only bind cdk4 or cdk6 (51). This interaction blocks recruitment of cyclin D to the complex. One explanation for the existence of four INK4 proteins may be that their expression appears to be differentially regulated. The $p15^{INK4b}$ gene is induced by transforming growth factor (TGF)-β, $p16^{INK4a}$ accumulates as cells age, and $p18^{INK4c}$ and $p19^{INK4d}$ are differentially expressed during embryonic development (for review, see 102). The interplay between p15 and p27 in Mv1Lu cells is a paradigm for how the INK4 proteins coordinate G1 growth arrest with Cip/Kip proteins. In response to TGF-β or contact inhibition, p15 levels increase in Mv1Lu cells and associate with cdk4 (95). The free p27 that becomes available as p15 sequesters cdk4 from cyclin D is now able to bind and inhibit cyclin E/cdk2 complexes (Fig. 3). In this case, the G1 restriction point is activated by the combined effect of INK4 inhibiting cyclin D/cdk4 and Cip/Kip blocking cyclin E/cdk2 kinase activity, resulting in retention of hypophosphorylated Rb and suppression of E2F transcriptional activity.

In addition to its ability to affect cdk activity, p21 is uniquely able to inhibit PCNA-dependent DNA elongation through its unique carboxy-terminal PCNA binding domain (72, 75, 122). The crystal structure of PCNA bound to a carboxy-terminal peptide of p21 suggests that p21 prevents recruitment of proteins required for DNA replication (49). Because p21 binds to the histone acetylase CBP/p300, it may also affect histone acetylation and therefore gene transcription (107). Indeed, p21 inhibits STAT3, Myc, and E2F transcriptional activity, suggesting that its growth inhibitory actions extend beyond just affecting cdk or PCNA activity (23, 26, 62). The dual role of Cip/Kip proteins at low levels to stimulate while inhibit at higher levels suggests that slight changes in expression can have dramatic effects on cell proliferation. As described below, increased or overexpression of p21 is associated with growth arrest. Growth inhibition by p21 is presumably achieved when it binds cyclin E/cdk2, PCNA, and/or key transcription factors required for DNA synthesis, and inhibits their activity.

Recent studies have led to a greater appreciation that p21 plays an important role in growth arrest of cells oxidized by hyperoxia or hydrogen peroxide. In SV40-transformed rat type II epithelial cells, hyperoxia increased p21 and inhibited cyclin E-dependent kinase activity (24). Although p27 mRNA increased, p27 protein remained unaltered and was not detected in cyclin E complexes. Additional studies in a number of nontransformed epithelial cells confirmed that hyperoxia increased p21 and exerted G1 growth arrest (54, 93, 101). As expected, cells that failed to express p21 exited G1 and ar-

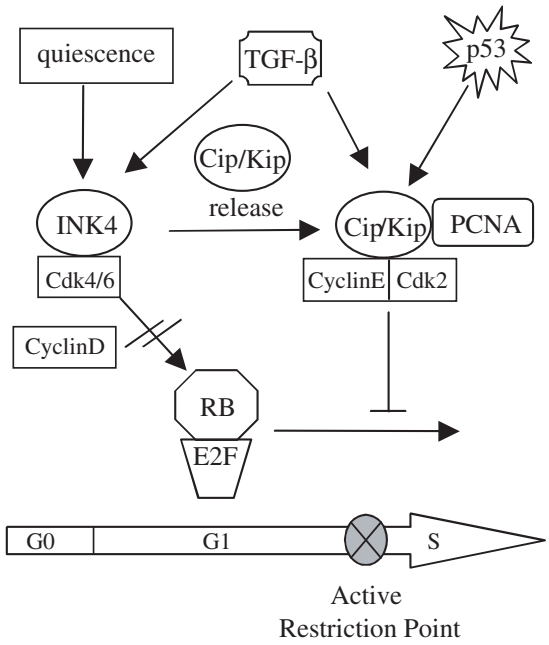

FIG. 3. Activation of the G1 restriction point by Cip/Kip and INK4 proteins. Growth arrest involves induction of INK4 proteins that associate with cdk4 or cdk6, thereby preventing cyclin D and cdk interactions. Cip/Kip proteins are released and free to inhibit cyclin E/cdk2 kinase. Rb is retained in the active growth inhibitory conformation and associates with E2F.

rested in S and G2 phases. Recently, overexpression of the amino- and carboxy-terminal domains in p53-deficient H1299 cells revealed that both domains were capable of individually restoring G1 control during hyperoxia (55). Moreover, the carboxy-terminal domain appeared more effective than the amino-terminal domain at limiting S-phase progression. In HCT116 colon carcinoma or H1299 lung adenocarcinoma cells, hyperoxia did not alter mRNA expression of p27, p57, or any of the INK4 genes (54, 55). Although these studies suggest that hyperoxia inhibits cyclin E/cdk2 solely by increasing p21, decreased cdk2 activity has been reported in T47D human breast carcinoma cells that exhibit reduced p21 during exposure (9). Like hyperoxia, hydrogen peroxide stimulated p21 expression and inhibited proliferation of IMR-90 fibroblasts (17), and as pointed out earlier, as radiation-induced DNA damage requires oxygen, it is not surprising that radiation inhibits proliferation in G1 via induction of p21 (12). These findings taken together reveal that oxidative stress inhibits proliferation in G1 by stimulating p21 expression.

p21 and DNA repair

Because DNA replication and repair are tightly linked processes, p21 is likely to participate in DNA repair. In this capacity, p21 may participate in long-patch base excision repair (BER) or nucleotide excision repair (NER) through its interactions with PCNA, a component of both processes. Unfortunately, it remains controversial how p21 affects repair. For example, it is well established that *p21*-deficient mice and cell lines are acutely sensitive to DNA damaging agents such as IR, cisplatin, nitrogen mustard, ultraviolet (UV) (27, 33, 76, 100), and hyperoxia (54, 86). Reporter plasmids damaged by alkylation or UV are more efficiently repaired in cells that express p21 than cells that do not. Similarly, regulated overexpression of p21 in *p53*-deficient H1299 cells afforded resistance to IR-induced toxicity (124). Because p21 also inhibits DNA replication, it has been difficult to determine whether p21 protects by simply blocking replication of damaged DNA or actually participating in DNA repair. For example, Mv1Lu mink lung epithelial cells growth arrest in S phase when exposed to hyperoxia, presumably because they fail to induce p21 (93). In contrast, cells may be retained in G1 by increasing density, reducing serum, or adding TGF-β. Unlike keratinocytes where TGF-β promotes G1 arrest by inducing p21, TGF-β induces G1 arrest in Mv1Lu cells by stimulating p27 activity (90). Although p21 is not expressed, Mv1Lu cells arrested in G1 exhibit significantly less DNA damage and greater survival than cells arrested in S phase (94). Although this study suggests that growth arrest in G1 promotes repair, it remains unclear whether adding p21 would augment the protective effects of G1 alone.

Other studies, however, have argued that p21 inhibits repair. For example, a gene array study using HT1080 fibrosarcoma cells revealed that p21 repressed expression DNA repair enzymes involved in NER (15). This finding is consistent with other studies showing that the PCNA binding domain of p21 blocked NER in cultured cells (22, 104). Several other proteins, including flap endonuclease (FEN)-1 and xeroderma pigmentosum (XP) G, compete for the p21 binding site on PCNA (37). FEN-1 is an essential component of DNA replication and long-patch BER because it excises flap structures. PCNA can stimulate BER through stabilizing FEN-1 on DNA (38, 116). Interestingly, mutants of PCNA that cannot bind FEN-1 are unable to stimulate excision repair, suggesting that p21 may prevent repair by blocking association of PCNA and FEN-1. The overwhelming abundance of data showing that p21 can repress or promote DNA repair strongly indicates that it may do both *in vivo*. Perhaps the level of p21 may dictate whether repair occurs or not. For example, low levels of p21 that promote proliferation may repress repair, whereas higher levels that inhibit proliferation may promote repair.

p21 and apoptosis

Consistent with its ability to promote survival by regulating DNA replication and repair, p21 has also been found to antagonize apoptosis. For example, two populations that can be discriminated as apoptotic with activated caspase 3 or growth-arrested and expressing p21 have been detected when human fibroblasts are exposed to low levels of hydrogen peroxide (17). Although the evidence is indirect, it suggests that p21 blocks apoptotic signaling. p21 also protects colon carcinoma and melanoma cells against prostaglandin A2 and p53-mediated apoptosis signaling (46, 47). One mechanism by which p21 might promote survival is by inhibiting apoptotic signaling by p53. Indeed, mouse embryo fibroblast cells lacking p21 express higher levels of Bax and are more susceptible to hydrogen peroxide killing (131). Likewise, loss of p21 in colon carcinoma cells increased sensitivity to chemotherapeutic drugs and was attributed to elevated p53 and Bax (56). Consistent with p21 protecting against DNA damage, *p21*-deficient mice exhibit increased sensitivity to ionizing radiation or hyperoxia (86, 125). It appears that p21 does not alter p53-dependent expression of Bax during hyperoxia because Bax mRNA and protein are not elevated in hyperoxic *p21*-deficient mice (O'Reilly, unpublished observations). Recent studies have led to a greater appreciation that cytoplasmic forms of p21 block apoptosis. During monocytic differentiation, cytoplasmic forms of p21 interact with and block apoptosis signal-regulated kinase (ASK)-1 (2). p21 also binds and blocks caspase 3 activation, thereby preventing Fas-dependent apoptosis (110). The binding of p21 with caspase 3 occurs in mitochondria, again suggesting that nonnuclear forms of p21 exert potent antiapoptotic properties. Cytoplasmic forms of p21 that interact with stress-activated kinase have also been detected during neuronal differentiation (111). p21 also participates in H-ras-mediated loss of actin stress fibers by blocking Rho kinase (68). Taken together, these findings reveal a novel role of cytoplasmic forms of p21 to affect cell survival and transformation.

Given that p21 antagonizes apoptotic signaling, it is not surprising to discover that multiple mechanisms exist to block its protective effects during apoptosis. The cytoprotective effects of p21 to suppress both growth and apoptosis are lost when it is attacked by caspases (41, 134). A caspase 3-sensitive site has been identified in the carboxy-terminus of human p21 that is cleaved early during apoptosis (89). As the caspase 3 site is not present in rat or mouse p21, it remains unclear whether alternative proteolytic mechanisms exist to degrade p21 during apoptosis. Proteolysis is not the only mechanism by which cells may override the protective effects of p21. The proto-oncogene c-Myc inhibits p21 expression by blocking binding

of p53 to the p21 promoter (99). Although not formally tested in that study, unbound p53 may be more available to increase proapoptotic genes such as Bax, Puma, or Noxa (34, 120). Although loss of p21 is associated with cell death, a recent study provided evidence that its degradation was required for optimal DNA repair following UV irradiation (7). Additional experiments are needed to understand how loss of p21 signals apoptosis versus repair.

REGULATION OF p21 EXPRESSION

p53-dependent expression of p21

The tumor suppressor p53 is arguably the predominant regulator of p21 when cells are damaged. Under nonstressed conditions, p53 binds to the E3 ligase murine double minute (mdm) 2, which targets both p53 and itself for ubiquitination. In this capacity, mdm2 promotes export of p53 from the nucleus to the cytoplasm where both are degraded by the proteasome (112). Cellular stress associated with DNA damage, altered redox state, or nutrient depletion induces p53 (82, 132). In response to DNA strand breaks, the phosphatidylinositol kinase-related kinase ataxia telangiectasia mutant (ATM) kinase becomes activated and phosphorylates p53 and mdm2 (3, 13, 61). Serine 15 phosphorylation on p53 enhances p53 transcriptional activity by promoting association with the p300 coactivator and possibly by blocking association with mdm2 (29, 103). As expected, *ATM*-deficient mice exhibit attenuated p53 and p21 induction in response to IR (5). Maintaining p53 activity requires activation of ATR, an ATM- and Rad3-related kinase (20). Although ATM and ATR are required for activating p53 following IR, only ATR is required to activate p53 following UV damage (105, 115). In HCT116 colon carcinoma cells, radiation and hyperoxia both stimulate serine 15 phosphorylation and p53-dependent induction of p21 (54, 123). Although IR stimulates p53 through ATM kinase, a recent study using *ATM*-deficient fibroblasts and dominant-negative ATR expression constructs concluded that hyperoxia activated p53 exclusively through ATR (25). Hydrogen peroxide also promotes p53 phosphorylation at serines 9, 15, and 20, of which the polo 3-like kinases have been implicated in phosphorylating serine 20 on p53 in an ATM-dependent manner (127, 128). Thus, different oxidants that damage DNA use unique kinases to activate p53. Presumably, the various lesions formed under different oxidant stresses activate unique kinases that phosphorylate and enhance p53 stability.

Cellular stress that does not damage DNA may also induce p53-dependent expression of p21. For example, ribonucleotide biosynthesis inhibitors, such as *N*-(phosphonoacetyl)-L-aspartate, that starve cells of nucleotide precursors induce p53-dependent expression of p21 and promote G1 growth arrest (70). Hypoxia also promotes p53-dependent expression of p21 without damaging DNA (52). Although hypoxia increased p21, intriguingly, it did not affect proliferation of human breast epithelial MCF-7 or IMR-90 fibroblasts (14). Hypoxic induction of p53 was ROS-dependent because MCF-7 cells depleted of mitochondria or treated with rotenone, a complex I inhibitor, failed to induce p53. Intriguingly, the same study showed that MCF-7 cells arrested in G1 with increased levels of p53 and p21 when exposed to IR. Although it remains unclear why p21 failed to elicit G1 delay during hypoxia but did following IR, it is clear that cellular stress leads to increased expression of p53 and p21 independent of whether DNA lesions can be detected.

Sequence comparison of the mouse, rat, and human p21 promoters revealed conservation of two p53-binding sites, of which the predominant site lies distally at –2.2 kb (Fig. 4) (31). *In vitro* oxidation can alter p53 conformation and inhibit DNA binding (50). Oxidized forms of p53 are reduced by redox factor–1 (Ref-1), which also reduces oxidized forms of activator protein-1 (AP-1) and nuclear factor-κB (57). Overexpression of Ref-1 enhanced p53 activity toward the p21 promoter, whereas antisense Ref-1 inhibited p53 activity (35). Ref-1 activity toward p53-dependent transcription of p21 may be enhanced by the dithiol-reducing enzyme thioredoxin, whose expression increases when cells are oxidized (81, 119). Thus, thioredoxin and Ref-1 maintain p53 transcriptional activity toward p21 under conditions that oxidize proteins. Given that p21 protects against apoptosis, one might hypothesize that these proteins might also affect apoptosis. Like p21, thioredoxin also directly binds and inhibits ASK-1 (98). However, p53-independent pathways also exist that may have evolved to ensure p21 expression under severe oxidant conditions that overwhelm thioredoxin or Ref-1 activity.

p53-independent expression of p21

A large number of p53-independent pathways also exist, some of which may have evolved to ensure p21 expression

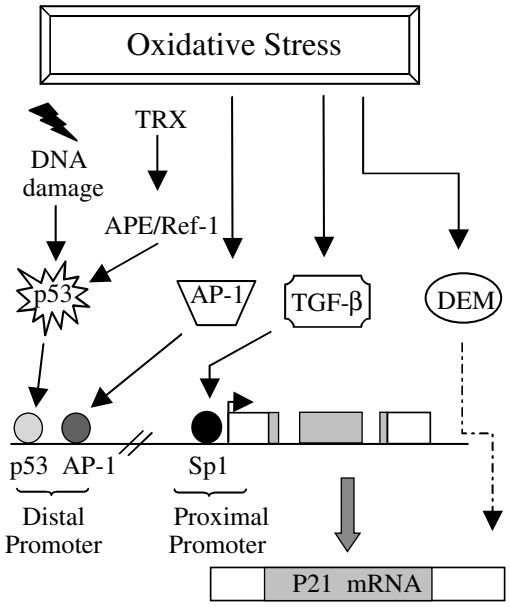

FIG. 4. Multiple pathways interface to regulate expression of p21. Oxidative stress activates a number of DNA damage-dependent and -independent pathways that increase transcription (solid arrows) or posttranscriptional stability (dotted arrow) of p21. The p21 gene contains three exons of which most of the protein is encoded in the second exon (filled boxes). APE, apurinic/apyrimidinic endonuclease; TRX, thioredoxin.

under severe oxidant conditions that overwhelm the cell's ability to maintain p53 activity. These pathways largely activate proximal *cis*-acting elements located within the first 120 bp of the promoter, a region that contains six Sp1 binding sites. The differential use of these sites is responsible for p21 induction by a number of mechanisms, including TGF-β, phorbol esters, retinoic acid, butyrate, histone deacetylase inhibitors, interleukin (IL)-6, protein kinase C, and the breast cancer susceptibility gene (BRCA1). These and other pathways have been reviewed in great detail (36).

The effects of oxidative stress and induction of p21 by TGF-β are particularly interesting because TGF-β expression is itself regulated by oxidants. TGF-β is initially synthesized as a larger proprotein that is cleaved upon secretion, resulting in an amino-terminal prodomain [latency-associated peptide (LAP)] and a carboxy-terminal TGF-β peptide. The secreted TGF-β remains noncovalently bound to LAP, thereby preventing receptor binding. TGF-β may be activated by altering pH and temperature (39), binding to β6 integrin (80), or oxidation of the latent complex (4). Once activated, TGF-β signals through cell-surface receptors to Smad proteins that partner with Sp1 to induce p21 transcription (79, 88). Mitogen-activated protein kinases, particularly c-Jun, may augment p21 expression by enhancing Sp1 activity (60). Thus, extracellular oxidative stress could induce p53-independent expression of p21 via TGF-β signaling. Indeed, neutralizing antibodies against TGF-β blocked hyperoxia-induced p21 expression in rat type II epithelial cells (24). Although this suggests that TGF-β signaling is required for hyperoxia to induce p21, p53 is required in other cell lines (54, 55). These disparate findings are actually consistent with *in vivo* observations showing p53-dependent and -independent induction of p21 in adult mice exposed to hyperoxia (77, 86). Not only can oxidants promote TGF-β-dependent expression of p21, but TGF-β can promote hydrogen peroxide production in fibroblasts by stimulating NADH oxidase expression (113). Thus, oxidants activate TGF-β, which in turn can stimulate p21 and ROS production.

Like hyperoxia, hydrogen peroxide also stimulates p53-dependent and -independent expression of p21. In *p53*-deficient H1299 cells, hydrogen peroxide induced p21 transcription through a novel AP-1 binding site located at –2.2 kb (19). In addition, extracellular signal-regulated kinase (ERK) stimulated AP-1 activity at this site. Intriguingly, hyperoxia does not induce p21 in H1299 cells, implying that hydrogen peroxide may not be the predominant ROS produced during hyperoxia (55). Other forms of oxidative stress can induce p21 expression. Diethyl maleate (DEM), which oxidizes cells by depleting reduced levels of glutathione, induced p21 expression via the ERK pathway (97). Unlike hydrogen peroxide that stimulated p21 transcription, DEM increased p21 expression by enhancing posttranscriptional stability of p21 mRNA (32). Thus, a large number of signal transduction pathways activated by oxidants converge at the level of p21.

FUTURE DIRECTIONS

The lung is an interesting organ to study the cellular response to oxidative stress because inhaled gases and particles often oxidize pulmonary cells. Inflammatory cells recruited to sites of injury may add to the oxidant burden. The clinical use of mechanical ventilation with assisted oxygen further stresses the lung. As such, it is not surprising that elevated levels of p21 have been reported in patients with asthma (92) or idiopathic pulmonary fibrosis (64). Cigarette smoke extract increases p21 expression in cultured human lung epithelial cells (73). p21 has also been detected in mice that develop bleomycin-induced pulmonary fibrosis (78). As pointed out several times in this review, hyperoxia increases p21 levels in the intact lung and in cultured cell lines (77, 85). Like radiation-induced damage, *p21*-deficient mice are highly sensitive to hyperoxia (86). Although the protective effects of p21 may lie in its ability to prevent proliferation of oxidized cells, the mitotic index of the adult lung is very small. Alternatively, p21-mediated protection may lie in its ability to affect apoptosis or DNA repair. Transgenic adult mice that overexpress IL-6 exhibit increased tolerance to hyperoxia (126). As IL-6 can regulate p21 expression (6), it remains unclear whether IL-6 protects by stimulating p21 expression. Similarly, activated Akt protects mice from hyperoxia (71). Akt is a pro-survival phosphatidylinositol-kinase that stabilizes p21 by phosphorylating its carboxy-terminus (69). As *p21*-deficient mice recovering from hyperoxia exhibit enhanced cell proliferation and myofibroblast hyperplasia (108), p21 may promote normal remodeling by controlling the rate at which injured cells replicate. In an analogous manner, the growth inhibitory actions of p21 may protect the developing lung from oxidative damage. Indeed, hyperoxia increased p21 in newborn mice and p21 was detected in nonproliferating cells of premature baboons that develop chronic lung disease (77, 87). Although speculative, p21 may protect neonatal cells from oxidative damage caused by premature exposure to oxygen, albeit at the expense of inhibiting normal lung growth and differentiation.

SUMMARY

Although p21 is a small 164-amino acid protein, it is obviously a major player in the complex cellular response to various oxidant stresses. Despite a decade of research, mostly focusing on how p21 inhibits DNA replication, it is only recently that new roles for this protein are being defined. Clearly, p21 merited study because it was the predominant downstream target of p53-dependent growth inhibition. At first glance, it seemed simplistic that all cells regulated p21 through p53. However, this naive viewpoint was reexamined as p53-independent activation and new roles for p21 were identified. A close examination indicates that oxidative stress induces p21 via a number of distinct pathways that regulate p21 at the level of transcription, posttranscriptional mRNA stability, and perhaps even posttranslational stability. p21 in turn regulates DNA replication, DNA repair, and apoptosis. Given the complexity of p21 expression and function in cultured cell lines, one can only imagine what happens in a more complex heterocellular tissue such as the lung. The challenge of the future will be to understand how these apparently divergent processes in different cells are coordinately coupled to ensure normal tissue function. A better understanding of how p21 modifies the cellular and tissue responses to oxidative stress could pro-

vide new therapeutics for a large number of diseases where ROS are implicated.

ACKNOWLEDGMENTS

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ABBREVIATIONS

AP-1, activator protein-1; ASK-1, apoptosis signal-regulated kinase; ATM, ataxia-telangiectasia mutant; ATR, ATM- and Rad3-related kinase; BER, base excision repair; cdk, cyclin-dependent kinase; Cip/Kip, cyclin-dependent kinase interacting/kinase inhibitory protein; DEM, diethyl maleate; ERK, extracellular signal-regulated kinase; FEN-1, flap endonuclease-1; IL, interleukin; INK4, inhibitors of cdk4; IR, ionizing radiation; LAP, latency-associated peptide; mdm2, murine double minute 2; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; Rb, retinoblastoma gene product; Ref-1, redox factor-1; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; UV, ultraviolet.

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