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Forum Review

Redox Regulation of Cell-Cycle Re-entry: Cyclin D1 as a Primary Target for the Mitogenic Effects of Reactive Oxygen and Nitrogen Species

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ABSTRACT

Reactive oxygen and nitrogen species inhibit or promote cell proliferation by modulating the cell signaling pathways that dictate decisions between cell survival, proliferation, and death. In the growth factor-dependent pathways that regulate mitogenesis, numerous positive and negative effectors of signaling are influenced by physiological fluctuations of oxidants, including receptor tyrosine kinases, small GTPases, mitogen-activated protein kinases, protein phosphatases, and transcription factors. The same mitogenic pathways that are sensitive to oxidant levels also directly regulate the expression of cyclin D1, a labile factor required for progression through the G1 phase on the cell cycle. Because the transition from G0 to G1 is the only phase of the cell cycle that is not regulated by cyclin-dependent kinases, but rather by redox-dependent signaling pathways, expression of cyclin D1 represents a primary regulatory node for the dose-dependent effects of oxidants on the induction of cell growth. We suggest that expression of cyclin D1 represents a useful marker for assessing the integration of proliferative and growth inhibitory effects of oxidants on the redox-dependent signaling events that control reentry into the cell cycle. *Antioxid. Redox Signal.* 7, 741–751.

INTRODUCTION

The term "oxidant" encompasses a large category of diverse chemical compounds. In mammalian cells, the most ubiquitous oxidants are reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$). Reactive nitrogen species (RNS), including nitric oxide (NO) and peroxynitrite ($ONOO^-$), comprise another large class of biologically important oxidants. A number of recent reviews have provided comprehensive lists of potential sources of oxidant formation (35, 88). Physiologically relevant concentrations of oxidants can be generated intracellularly or formed exogenously and be taken directly up into the cell (35, 69).

ROS/RNS have the requisite properties required for effective mediators of cell signaling: they are generated in response to a variety of extracellular and intracellular stimuli, are diffusible in and between cells, and interact with specific molecular targets, and their reactivity and regulation by antioxidants ensure they are largely transient in nature (34, 65). It is now known that endogenously generated oxidants, rather than merely being toxic by-products of respiration and a mechanism for host defense by immune cells, are often used by the cell as indispensable mediators of signal transduction and transcriptional regulation (34, 38, 105). Depending on the concentration and the duration of exposure, ROS/RNS may increase cell proliferation, induce cell-cycle arrest, or induce cell death, through either necrotic or apoptotic mechanisms (69). There appear to be few cellular processes that do not respond to imbalances in ROS/RNS, and inappropriate regulation of cellular redox status and/or interaction of environmental oxidants with redox-dependent signaling pathways have been implicated in many acute and chronic diseases (35).

It has long been appreciated that low levels of ROS and RNS can dramatically affect the rate of proliferation, and this topic has been addressed in several excellent reviews (12, 18,

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36, 88, 92). Our purpose here is to provide a perspective on how ROS/RNS influence the G0 to G1 transition of the cell cycle, or the transition from quiescence to growth. We consider this a critical interval in cell-cycle progression because in adult vertebrate organisms, most cells have withdrawn from the cell cycle and are maintained in a quiescent state (G0), with only a few tissue compartments actively proliferating. Most cells in adult tissues reenter the cell cycle and resume proliferation only in response to tissue injury, or to replace cells lost during normal tissue function. We are intrigued by the fact that the G0 to G1 transition is the only transition of the cell cycle that is not regulated by cyclin-dependent kinases (CDKs), but rather by redox-sensitive cell signaling and gene expression cascades (Fig. 1). If control of this transition is indeed dependent on signaling by ROS/RNS, then, as for all signaling mechanisms, there must be molecular targets that promote cell-cycle progression at physiological levels of oxidants, and targets that prevent progression when ROS/RNS levels exceed a critical threshold. Hence, one might expect the cell to utilize multiple mechanisms to modulate ROS/RNS levels in order to balance signaling decisions between proliferation, arrest, and apoptosis. Loss of this balance is evident from the involvement of ROS in neoplastic transformation, which involves many oncoproteins that also regulate or are regulated by ROS/RNS, such as Ras, Raf, c-Jun, c-Fos, and p65 RelA. Interestingly, all of the pathways that promote mitogenesis through ROS/RNS also converge at the level of transcription of the cyclin D1 gene, which serves as a functional marker for exit from G0 and entry into G1. Once cells are actively cycling, ROS may encourage proliferation in normal cells by maintaining cyclin D1 levels, and contribute to neoplastic transformation by bypassing the requirement for growth factors in mitogenic signaling.

RECENT REVISIONS IN THE UNDERSTANDING OF CELL-CYCLE CONTROL

Cell-cycle progression is a highly ordered process that ensures the faithful replication of a cell's genome and division into two daughter cells. Initially informed by work in yeast, studies in animal cells have generated a widely accepted model in which progression through the cell cycle is regulated by the stepwise synthesis and degradation of a series of cyclin gene products. Together with their cognate CDKs, D- and E-type cyclins cooperate to drive the cell through the G1 phase of the cycle via modulation of the retinoblastoma (pRB) protein pathway and assembly of replication complexes through a process termed origin licensing (15, 94). Progress through the G1 phase of the cycle is monitored by CDK inhibitors such as p21 and p27, as well as checkpoint proteins such as p53 (Fig. 1). These and other

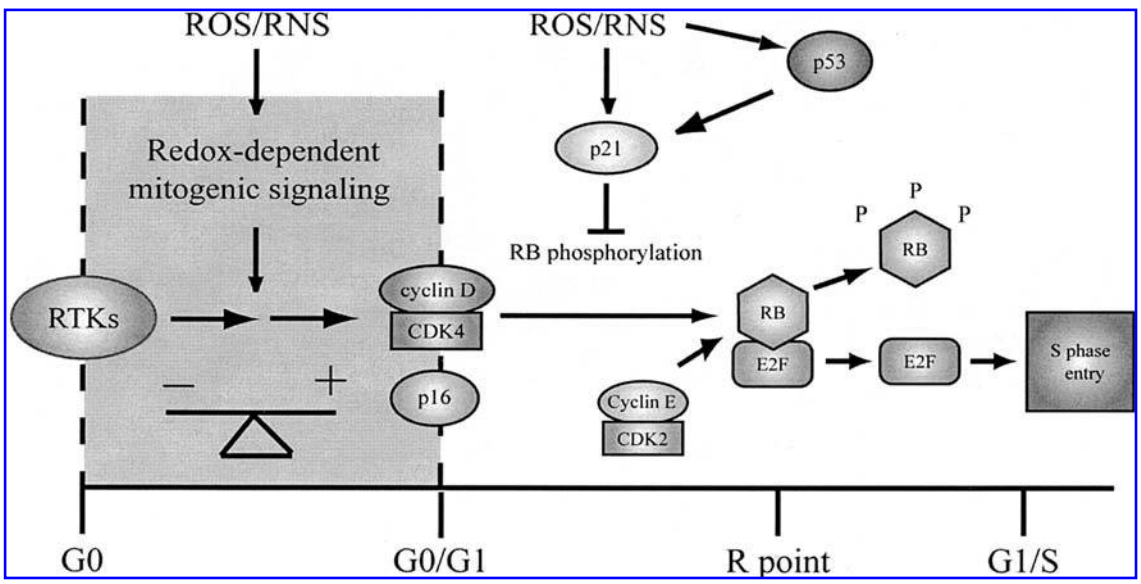

FIG. 1. The redox status of the cell influences the outcome of mitogenic signaling during cell-cycle reentry. The G0 to G1 transition is the only phase of the cell cycle that is not regulated by CDKs, but rather by redox-dependent cell signaling pathways. Exposure to environmental oxidants or the production of endogenous oxidants influences mitogenic signaling in quiescent cells in both a positive and negative manner, depending on the dose and duration of the intracellular oxidant levels. During cell-cycle reentry, the successful integration of mitogenic signals from multiple sources, including growth factor receptors, extracellular matrix, and actin cytoskeleton, is reflected in expression of cyclin D1. High levels of oxidants impede the completion of mitogenic signaling and induce cell-cycle arrest in G0 upstream of cyclin D1 expression. After entry into the cell cycle, actively cycling cells monitor the redox status of the cell through p53-dependent and -independent damage-sensing mechanisms, resulting in cell-cycle arrest by inhibition of CDK activity. However, in certain cells, oxidants may inhibit withdrawal from the cycle by promoting activation of Ras in G2, which is required for maintaining expression of cyclin D1. Fluctuations in the levels of cellular antioxidants also govern oxidant levels, providing additional cell type-specific mechanisms for modulating redox-dependent signaling.

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factors ensure that cells arrest prior to S phase if damage to DNA or other macromolecules is detected (32, 95). In a similar fashion, progression through S phase requires A-type cyclins; completion of mitosis requires B-type cyclins. These phases of the cycle have their own unique checkpoint pathways (32, 94).

Recent work in knockout mouse models has disturbed the foundation of this well established model for cell-cycle control. Mice lacking D-type cyclins are viable with modest developmental defects, although smaller than their littermates (24, 33, 98). Viability was thought to be due to compensation by cyclin E in pRB phosphorylation, but surprisingly, as long as they are provided with a normal placenta (via injection of embryonic stem cells into tetraploid blastocysts), animals lacking E-type cyclins also develop to term with relatively minor defects in the cardiovascular system (39). Similarly, CDK2, the CDK partner of cyclin E, is not required for entry into the S phase in colon cancer cells (108), and mice lacking CDK2 are viable and fertile (80).

Although analysis of these models indicates that other CDKs may compensate for loss of CDK2, the cyclin E knockout result was truly unanticipated because studies *in vitro* had shown that expression of E-type cyclins is absolutely required for initiation of DNA synthesis, centrosome duplication, histone gene expression, and other molecular events leading to S phase. Indeed, mouse embryo fibroblasts derived from animals lacking E-type cyclins cannot reenter the cell cycle *in vitro* due to defects in recruitment of replication factors to chromatin during origin licensing (39). Continuously cycling mouse embryo fibroblasts deficient in cyclin E do not, however, display this same failure in origin licensing, suggesting cyclin A may be able to compensate for the loss of cyclin E in actively proliferating cells and, thereby, provide a possible explanation for why cyclin E knockout mice develop normally. Together these remarkable results indicate that the G1 CDK pathway is not required in any tissue during embryogenesis for proliferation and differentiation, and imply that there is a great deal left to learn about the regulation of cell proliferation during development. In contrast, lineage tracing and other studies show that regeneration of certain cell types in adult animals clearly requires exit from G0 and reentry into the cell cycle (31). We suggest that it is these pathways that are the primary target of oxidants in cell proliferation.

CYCLIN D1 CONTROLS THE G0 TO G1 TRANSITION

Early experiments on cells in culture exposed to oxidants revealed an entire spectrum of proliferative responses, including increased proliferation, transient cell-cycle arrest, permanent senescence, and cell death, depending primarily on the relative dose of exposure (27, 69). The mechanisms of cell-cycle arrest often involve the activation of stress and damage-activated signaling pathways due to the toxic effects of high doses of oxidants (69). For example, oxidants induce expression of the CDK inhibitor p21 through both p53-dependent and p53-independent mechanisms (11, 20, 23, 29, 117). Prolonged arrest in G1 or S phase due to damage to macromolecules by high levels of ROS/RNS is often followed by apoptosis, again by p53-dependent and p53-independent mechanisms (55, 67, 69).

Expression of inhibitory proteins that act through cyclin/CDK complexes in response to oxidants would be expected to have little effect during the G0 to G1 transition because the molecular targets for such inhibitors are irrelevant at this phase of the cell cycle. Indeed, expression of the CDK inhibitor p21 is required for the assembly and nuclear entry of active CDK complexes during cell-cycle reentry (83, 96). How then do low doses of oxidants promote transition from G0 to G1, and higher doses impede it? Our recent studies suggest expression of cyclin D1 represents a redox-dependent regulatory node by which oxidants act to both encourage and block reentry into the cell cycle.

A critical step in tissue regeneration occurs at the level of mitogenic stimulation, which acts to initiate reentry into the cell cycle. The D-type cyclins, especially cyclin D1, form a critical link between the initiation of mitogenic signaling pathways and activation of the cell-cycle machinery in most normal cell types. In actively proliferating cells, cyclin D1 transcript levels do not vary much and cellular cyclin D1 protein levels are primarily controlled by posttranslational mechanisms. In the G2 phase of cycling cells, extracellular levels of growth factors that act through Ras dictate whether the cell will continue to cycle upon exit from mitosis, or withdraw from the cycle into G0 (45, 99). Upon withdrawal from the cycle, repression of transcription and protein degradation lead to marked reductions in cyclin D1 levels in most cell types, and levels remain low until cells reenter the cycle. In both terminally differentiated cardiomyocytes and postmitotic neurons, cyclin D1 levels remain elevated, but the protein is sequestered in the cytoplasm by the inhibition of the nuclear import and therefore is incapable of driving cell-cycle progression (102, 106).

A central role for cyclin D1 in cell-cycle reentry is manifest in the fact that it is the only cyclin that can drive terminally differentiated cells back into the cycle (62), as well as the complexity with which cyclin D1 protein levels are regulated during mitogenesis. Transcription of cyclin D1 is dramatically up-regulated upon growth factor stimulation through the Ras/Raf/MEK/extracellular signal-regulated kinase (ERK) pathway. The protein is stabilized through activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and its downstream effectors (51, 56). Its assembly with CDK4 and entry into the nucleus are dependent on growth factors (for review, see 15).

Cyclin D1 transcription can be induced by numerous transcription factors that interact with binding sites located in the cyclin D1 promoter region (Fig. 2). The complexity of the cyclin D1 promoter is expected because the decision to express this gene reflects integration of signaling responses from multiple pathways, including those that monitor adhesion to an extracellular matrix (6, 73, 119), maintenance of the actin cytoskeleton (46), and activation of membrane-bound growth factor receptors (56, 84). The integration of signals at the level of cyclin D1 expression also is reflected in the fact that, in most cell types, it takes 4–6 h after mitogenic stimulation for cyclin D1-CDK4 activity to accumulate to sufficient levels to drive progression through G1. How then do oxidants influence the expression of cyclin D1?

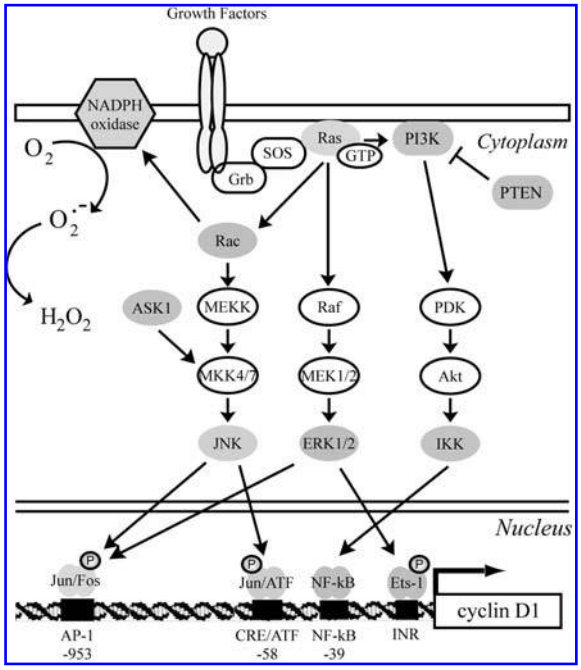

FIG. 2. The redox status of the cell influences cyclin D1 expression through several parallel signaling pathways. The primary signaling pathways that regulate cyclin D1 expression during the G0 to G1 transition of the cell cycle are known to be both positively and negatively regulated by the redox status of the cell. Signaling proteins that respond to or regulate the production of oxidants are shaded gray. Oxidants and growth factors, alone or in combination, induce activation of RTKs, resulting in the activation PI3K and Ras-dependent signaling pathways. Rac is a second small GTPase that has been shown to participate in the activation of both membrane-bound NADPH oxidase complexes and the JNK signaling pathway. The respiratory burst induced by mitogens has been demonstrated to modulate signaling through the ERK, JNK, and PI3K pathways, and all three pathways have been shown to contribute to the regulation of cyclin D1 transcription through redox-dependent transcription factors, including AP-1, NF-κB, and Ets proteins. We suggest that the promoter of the cyclin D1 gene represents a primary regulatory node for integrating both the positive and negative effects of oxidants on cell-cycle reentry.

PATHWAYS REGULATING EXIT FROM G0

As shown in Fig. 2, reentry into the cell cycle most often is initiated by the cooperative action of multiple mitogenic pathways, including the ERK and PI3K/Akt (51, 56). These mitogenic signaling pathways influence cyclin D1 transcription through coordinate activation of several families of transcription factors, including activator protein-1 (AP-1) (4, 9, 16, 43, 114), nuclear factor-κB (NF-κB) (41, 44, 57), Sp1 (63, 78, 111), cyclic AMP response element-binding protein (26, 78), ternary complex factor (97, 107), and others. Of these factors, AP-1, NF-κB, and Sp-1 have been shown to be regulated in a redox-dependent manner.

Mitogenesis usually begins with the interaction of cell type-specific growth factors with plasma membrane receptor tyrosine kinases (RTKs) (56, 109, 112). Activation of RTKs leads to tyrosine phosphorylation of the receptor, and recognition of these modifications by SH2, SH3, and other specific domains in signaling proteins mediates the assembly of complexes that transmit and amplify the mitogenic response. In response to platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or granulocyte/macrophage colony stimulating factor, a transient burst of intracellular H_2O_2 is produced during the mitogenic response (7, 30, 103). In these models, antioxidants have been shown to diminish or ablate the mitogenic response, showing that ROS are required for signaling to downstream targets of RTKs. Exogenous oxidants such as H_2O_2 added to culture media in low concentrations mimic the cellular production of ROS and, in some instances, induce increases in tyrosine phosphorylation in RTKs within minutes of exposure (88). Enhanced tyrosine phosphorylation by RTKs in response to oxidants is also promoted by transient oxidation of cysteines in the catalytic site of tyrosine phosphatases (115), thereby prolonging the maintenance of phosphorylated tyrosine residues.

The source of H_2O_2 in the mitogenic response is not entirely clear, but RTK activation has been linked in many instances with increased activity of NADPH oxidase complexes in the plasma membrane (82, 88). These complexes generate superoxide, which is rapidly converted to H_2O_2 by superoxide dismutase. Diphenyleneiodonium (DPI), a potent inhibitor of superoxide production from NADPH oxidases, interferes with mitogenic stimulation in several nonphagocytic cell types (1, 14, 86). Involvement of nonphagocytic NADPH oxidases in cell-cycle reentry also is supported by studies that show ectopic expression of Nox1 (originally termed Mox1) induces mitogen-independent cell growth and neoplastic transformation of NIH 3T3 cells through an H_2O_2-dependent mechanism (5, 101). Recent evidence indicates that Nox1 is required for transformation by oncogenic Ras and that this process is ERK-dependent (75). Expression of Nox1 in mouse lung epithelial cells also promotes proliferation, and this appears to occur through increased expression of cyclin D1 (P. Ranjan and N.H. Heintz, unpublished observations).

Whereas the mechanism of regulation of the gp91 phox (or nox2) oxidase complex of phagocytes by the small GTPase Rac2 (and in some cases Rac1) is well described (37), the details of the signaling pathways that regulate other nonphagocytic oxidases are still poorly understood. Generation of superoxide is required for mitogenesis in response to PDGF, and this is mediated by the PI3K/AKt pathway (8), which is capable of activating Rac1. Inactivation by oxidation of the tumor suppressor protein PTEN (phosphatase and tensin homologue on chromosome 10), a phosphatase that negatively regulates the PI3K/Akt pathway, also may contribute to activation of nonphagocytic oxidases by promoting activation of Rac1 (59). Although dominant negative Rac1 inhibits production of ROS in response to growth factors in NIH 3T3 cells (104), expression of constitutively active Rac in some cells causes increases in intracellular H_2O_2 that cannot be blocked by inhibitors of NADPH oxidases such as DPI (113). Novel proteins that activate superoxide production by Nox1 have been identified (10), but the role of these factors in mitogenic signaling has not yet been reported.

Another issue of interest is how signaling by the transient generation of H_2O_2 occurs in the presence of cellular antioxidants. Because of the relatively high K_m of the enzyme, catalase would not be expected to play a major role in modulating low levels of peroxide in the cell. Recently, a model has been proposed by Wood *et al*. that ascribes modulation of signaling activity to peroxiredoxins (Prxs), which are much more sensitive to inactivation by the substrate H_2O_2 in mammalian cells than in bacteria (116). Wood *et al*. propose a "floodgate hypothesis" in which the transient production of H_2O_2 rapidly inactivates Prxs, thereby allowing H_2O_2 to interact with other targets, as has been observed in cells treated with tumor necrosis factor (85). Consistent with this hypothesis is the observation that mitogen-activated protein kinase (MAPK)-dependent activation of gene expression through the antioxidant response element by Nox1-induced H_2O_2 production occurs without the oxidation of cellular glutathione or thioredoxin-1 (40). In the floodgate model, catalase and glutathione peroxidase act as a failsafe mechanism to prevent H_2O_2 from reaching cytotoxic levels. Inactivation of Prx1 by oxidation of the active-site cysteine is reversible, providing a mechanism for regenerating Prx activity as signaling terminates (115). The ability to reduce the oxidized, sulfinic acid form of Prx1 to the catalytically active thiol form represents a novel molecular switch that might be used by other redox-sensitive proteins in cell signaling cascades.

The small GTPase Ras is also implicated in redox-dependent signaling. NIH 3T3 cells that express a constitutively active form of Ras produce significant concentrations of ROS (47), a situation encountered in many tumor cells. Expression of activated Ras in primary cells, however, induces senescence, a phenotype that requires expression of the tumor suppressor proteins p53 and p16 (91). Low doses of H_2O_2 also induce growth arrest with some features of senescence, including increased expression of p53 and p21 (8). Although bypass of permanent arrest by activated Ras in normal cells via the inactivation of p53 is easily reconciled, it is not clear how p16 could be involved in mediating arrest in response to oxidative stress. It is interesting to note, however, that p16 levels increase dramatically during senescence (48), and that the targets of p16 are the cyclin D/CDK complexes that control progression into and through G1. In addition, it also is interesting that the other product of the p16 locus, p14 ARF, has been shown to form protein multimers in a redox-dependent manner through highly conserved cysteine residues (72), implying that regulation of this tumor suppressor protein is redox-dependent. Although there clearly is a relationship between the transformed phenotype, elevated levels of ROS and small GTPases in tumor cells, and ROS and cell-cycle arrest in normal cells, more detail concerning the molecular mechanisms that regulate these phenotypic outcomes is required.

Downstream of Ras in mitogenic signaling pathways lie the MAPKs, a family of serine-threonine kinases that include ERKs, c-Jun N-terminal kinases (JNKs), and p38 kinase. The activation of all three of the MAPKs have been demonstrated to be influenced by cellular stresses, and their activities have been linked to cellular outcomes that include proliferation, survival, and cell death (19, 52). The ERK and JNK pathways participate in the regulation of AP-1 (18, 58, 69, 100), the family of transcription factor complexes composed of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families of proteins. These transcription factors are of particular interest because AP-1 is intimately involved in control of proliferation and neoplastic transformation, and is readily activated by ROS/RNS.

In general, c-Jun is a target of JNKs, and c-Fos a target of ERKs, and both are activated by oxidants (28, 69, 87). However, c-Jun and c-Fos play multiple roles in cell processes such as proliferation, recovery from damage, and apoptosis. Once activated, JNK translocates into the nucleus where it phosphorylates c-Jun on serines 63 and 73 (28), modifications that promote transcriptional activation. Mice lacking c-Jun do not survive embryogenesis (54), and fibroblasts from c-Jun −/− mouse embryos have severe defects in proliferation that are due to a dramatic increase in the length of G1, a defect only partially rescued by expression of cyclin D1 (114). Interestingly, mice that express a mutant form of c-Jun in which serines 63 and 73 have been replaced with alanine residues are viable and fertile, but smaller than controls (13). These mice are resistant to certain forms of apoptotic stimuli, suggesting that JNK-dependent phosphorylation of c-Jun at serines 63 and 73 is linked to apoptosis rather than proliferation. This may explain why JunB, which is not a substrate for JNK, is able to compensate for c-Jun in development (93), although these mice die soon after birth.

Of all the Jun proteins, c-Jun has the most potent activity in cooperating with activated Ras in transformation assays (3, 89, 90, 110), a result that agrees well with the observation that c-Jun −/− cells are resistant to transformation (53). Analysis of AP-1 activity during exit from mitosis suggests that c-Jun acts as a positive effector of cyclin D1 expression, whereas JunB is a negative regulator (9, 22). Phosphorylation of c-Jun during entry into G1 combined with CDK1/cyclin B-mediated destruction of JunB acts to tip the balance of AP-1 complexes toward proliferation. Although there is little evidence that JNK regulates proliferation, it is possible that oxidants produced in response to growth factors may induce a pulse of JNK activity that promotes proliferation through phosphorylation of c-Jun.

In contrast to c-Jun, c-Fos is a direct target for phosphorylation by ERK (21, 76, 79). Interestingly, brief activation of ERK (15–30 min) in response to growth factors is not sufficient for proliferation, and this may be due to the decay of ERK signaling before c-Fos is expressed at high enough levels to promote AP-1-dependent transcription. Activation of ERK for longer periods first leads to stabilization of c-Fos and exposure of an ERK-docking domain that promotes phosphorylation of c-Fos on C-terminal sites that promote transcriptional activity and proliferation (77). This finding is of considerable import because it shows that the duration of ERK signaling is linked directly to outcome.

The duration of ERK signaling in response to exogenous H_2O_2 is therefore a matter of some interest. When added as a bolus to culture medium (usually at 200–300 μM), H_2O_2 increases ERK2 activation in many cell types and increases cell survival (42). Due to the short half-life of H_2O_2, ERK activation in this instance may be transient in nature and act in a positive fashion on cell proliferation. In situations where ROS levels are increased beyond a certain threshold, ERK activation may be prolonged and thereby contribute to apopto-

sis, a notion supported by a recent study that showed that activation of ERK1,2 through the Raf/Ras pathway is required for apoptosis in response to H_2O_2 in L929 mouse fibroblasts, whereas inhibition of JNK activity through multiple approaches has no effect (64). These studies were performed by exposing log phase cells to high concentrations of H_2O_2 (1–2 m*M*), a level that far exceeds that produced by the cell during mitogenic signaling.

In studies from our lab, we have used fluxes of various oxidants, including NO_2, peroxynitrite, and H_2O_2, and examined the effect on the transition from G0 to G1 in serum-stimulated lung epithelial cells. Low levels of oxidants cooperate with low levels of serum (or growth factors) to induce cyclin D1 expression in this model, a result of transient increases in ERK1,2 activity. Higher doses of oxidant, however, induce cell-cycle arrest. Surprisingly, this effect is related to the prolonged activation and nuclear retention of ERK1,2. Under these conditions, activated ERK and hyperphosphorylated c-Fos accumulate on chromatin, and Fra-1 does not enter the nucleus. Immunostaining shows that no cells with nuclear c-Fos express cyclin D1, whereas all cells that express nuclear Fra-1 also express cyclin D1, suggesting that the degradation of c-Fos and subsequent recruitment of Fra-1 to chromatin are required for expression of cyclin D1. Hence, we suspect that c-Fos acts indirectly in the regulation of cyclin D1 transcription, whereas Fra-1 may be a direct regulator, and that these proteins act as effectors of the ERK response to oxidant levels.

In serum-stimulated cells treated with asbestos, which activates ERK and induces both proliferation and apoptosis, those cells showing nuclear phospho-ERK after 6 h of stimulation show migration of apoptosis-inducing factor from the mitochondria to the nucleus. (Z. Yuan and N.H. Heintz, unpublished observations). These cells then die from apoptosis, whereas neighboring cells that lack c-Fos and nuclear phospho-ERK express cyclin D1 and proceed on to S phase. Although asbestos activates ERK, activation through the EGF receptor, up-regulates c-jun and c-fos mRNA, and increases AP-1 DNA-binding activity, the actual outcome in terms of proliferation versus apoptosis may be linked to the duration of ERK signaling, and/or to the time during the cell cycle when the signaling response is elicited.

This may help explain an interesting paradox of cell-cycle regulation. As mentioned above, continuous mitogenic signaling induced by the forced expression of constitutively activated Ras or Raf-1 in untransformed cells results in G1-phase cell-cycle arrest (66, 120). This arrest could be prevented by treatment with the MEK inhibitor PD98059, indicating that the continuous activation of the ERK pathway was the initiator of the arrest (66). As a transient burst of H_2O_2 by NADPH oxidase complex is an obligate mediator of ERK activation (103), it seems reasonable to speculate that continuous oxidant exposure may lead to an ERK-mediated cell-cycle arrest by blocking the transcriptional activation of the major cell-cycle regulatory target of the ERK pathway, cyclin D1 (17, 118). As for the studies of Lin *et al*. (66), cyclin D1 expression in our studies was partially restored by down-regulating the oxidant-induced ERK activation with the MEK inhibitor U0126 (17). Similar effects of oxidants on the expression of cyclin D1 have been described in fibroblast cells treated with H_2O_2 (11), and for cyclin E in epithelial cells exposed to hyperoxia (25).

Other studies also indicate that the primary mediator of the transcriptional block of cyclin D1 mediated by prolonged ERK activation is AP-1. Upon mitogen stimulation, the Fos family proteins in the AP-1 complex normally undergo a dynamic exchange of the immediate-early gene product c-Fos for the delayed-early gene product Fra-1 (60, 61, 70, 71). Fra-1 is the Fos family member whose expression is coincident with the onset of cyclin D1 expression ~4–6 h after mitogen stimulation, whereas c-Fos is normally exported from the nucleus and degraded by that point (2, 61, 79). In our studies, prolonged ERK activation results in the retention of c-Fos in the nucleus and prevents its degradation. Treatment with the MEK inhibitor U0126 induces the degradation of c-Fos and promotes recruitment of Fra-1 to the chromatin, indicating that this process is dependent on down-regulation of ERK activity. This result correlates well with the observation that ectopic expression of c-Fos inhibits cyclin D1 expression, principally through the AP-1 site located 945 bp distal to the transcription start site (4, 74). NF-κB has also been implicated in the regulation of cyclin D1 expression (41, 44, 57), and similar redox-dependent events in signal duration may influence the role of NF-κB activity in cyclin D1 expression (49, 50, 68, 69, 81), for we have also observed that oxidant exposure that blocks cyclin D1 expression causes the accumulation of p65 RelA on chromatin.

Clearly, the G0 to G1 transition is both activated and inhibited in a dose-dependent manner by oxidative stress, with diverse agents acting through the modulation of intracellular levels of H_2O_2. This is precisely the manner by which redox-dependent signaling in mitogenesis should work: transient signals elicit a cascade of cell signaling and gene expression events directly or indirectly through a plethora of redox-dependent targets (*e.g*., RTKs, MAPKs, phosphatases, and transcription factors). Reduction in the intracellular levels of ROS terminates these signals, leading to expression of cyclin D1, entry into G1, and progression into the S phase. Failure to terminate the signal blocks cells at a specific interval in G0 (*i.e*., with activated c-Fos bound to chromatin), and persistent idling in this state eventually leads to cell death, which in lung epithelial cells is mediated by apoptosis-inducing factor. It is not clear why evolution favors such a complicated mechanism for reentering the cell cycle, for one could imagine that expression of cyclin D1 could be linked directly to growth factor activation. Perhaps cells need a period of time to integrate signals concerning cell size, adhesion to a substrate, the status of the cytoskeleton, and cell-to-cell contact before committing to cell division. Clearly, it would be useful to know the precise targets of c-Fos during G0, as well as the genes in addition to cyclin D1 that are regulated by AP-1 after degradation of c-Fos.

Finally, exposure of cycling cells to oxidants elicits responses that include induction of AP-1 activity, cell-cycle arrest, and apoptosis. The array of genes that are able to be activated by selected transcription factors is surely different in G0 than in other phases of the cell cycle, so activation of the same signaling pathways may lead to expression of a different subset of genes. Because these gene subsets may be cell type-specific, as well as cell cycle-specific, activation of the same

signaling pathways by oxidants could be used to drive different outcomes at the level of gene expression. Carefully designed experiments to assess oxidant-dependent expression profiles of different cell types poised at different phases of the cell cycle would identify these gene subsets, and thereby provide additional insight into the role of ROS and RNS in cell proliferation, survival, and death.

ABBREVIATIONS

AP-1, activator protein-1; CDK, cyclin-dependent kinase; DPI, diphenyleneiodonium; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, ERK kinase; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; pRB; retinoblastoma protein; Prx, peroxiredoxin; RNS, reactive nitrogen species; ROS, reactive oxygen species; RTK, receptor tyrosine kinase.

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