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Signaling to p53: Breaking the MDM2–p53 Circuit

Minireview

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The p53 tumor suppressor protein transmits signals arising from various forms of cellular stress, including DNA damage, hypoxia, and nucleotide deprivation, to genes and factors that induce cell cycle arrest and cell death. We can thus define events as being upstream or downstream of p53. The endpoint of the upstream component of this pathway is that levels of the p53 protein are dramatically increased through posttranscriptional mechanisms, often by one or two orders of magnitude. There is also evidence that in addition to elevating p53 levels, such signals convert the protein from an inert form to one that is activated for sequence-specific transcriptional activation (reviewed in Ko and Prives, 1996). Stress signals are not the only means by which p53 becomes stabilized. The DNA viruses SV40 and adenovirus encode gene products, T antigen and E1a, respectively, that lead to increased quantities of p53 protein in cells. Moreover, under some conditions expression of cellular factors such as Myc or Ras can also result in p53 induction. Although this might seem at odds with the outcome of transformation caused by viral or cellular oncogenes, viruses and cells have evolved several ways to counteract the growth suppression functions of induced p53. The downstream component of the p53 pathway has been relatively well explored, and many transcriptional targets of p53 have been identified and characterized, including the gene encoding the CDK inhibitor, p21. By contrast, the upstream component had been quite elusive until recently, and a number of significant breakthroughs have now provided insight into this aspect of p53. While there are likely to be multiple modes of induction of p53, current knowledge suggests that diverse upstream signals funnel into a single critical interaction, namely that between p53 and its negative regulator, MDM2.

p53 and MDM2

The ability of p53 to either restrain or kill a cell must be reigned in under normal conditions. MDM2 accomplishes this in two ways: as a result of its physical interaction with p53, MDM2 both represses p53 transcriptional activity and mediates the degradation of p53. The latter function of MDM2 was revealed when it was discovered that overexpression of MDM2 results in reduced quantities of coexpressed p53, and that disruption of the p53–MDM2 interaction by mutation results in both activation and accumulation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). The region on p53 with which MDM2 interacts (residues 17–27) is interesting for several reasons: First, it is one of the segments of p53 that is highly conserved not only among different species, but even among some p53-related family members, such as p73. Second, this portion of p53 is located within the transcriptional activation region, which is required for the interaction of p53 with components of the general transcriptional machinery, such as TAFs. Third, a number of phosphorylation sites have been identified in the vicinity of this region that are highly likely to be involved in regulating p53.

How does MDM2 mediate p53 degradation? There have been a number of attempts to address this question. Honda et al. (1997) reported that MDM2 can function as an E3 ubiquitin ligase, a result that is consistent with evidence that MDM2 destabilization of p53 is reduced in the presence of proteasome inhibitors (Haupt et al., 1997; Kubbutat et al., 1997). Grossman et al. (1998) have provided evidence that MDM2 needs to bind to the p300 transcriptional coactivator/histone acetylase in order to mediate degradation of p53. Adding further complexity to this issue, Roth et al. (1998) have shown that MDM2 shuttles p53 from the nucleus to the cytoplasm where it is then degraded. We can undoubtedly expect further clarification of the mechanism of MDM2-mediated degradation of p53 in the near future.

Given the above information, it has become clear that one way to stabilize and activate p53 in cells is by interfering either with the interaction between MDM2 and p53 or with the ability of MDM2 to target bound p53 for degradation. How might the p53–MDM2 interaction be regulated? At least two mechanisms can be envisaged, one through changes in p53 due to covalent modification, and the other through noncovalent regulators of the p53–MDM2 association. It is now apparent that both mechanisms can be identified under experimental conditions: DNA damage–induced phosphorylation of p53 can attenuate the p53–MDM2 interaction, and the product of the alternate reading frame (ARF) located within the *p16*^{INK4A} locus (murine p19^{ARF}, human p14^{ARF}) can bind to MDM2 and prevent its destruction of p53. Interestingly, these two mechanisms appear to be entirely separate and independent from each other, and regulated through distinct signaling pathways.

Signaling through Covalent Modification of p53

The phosphorylation status of a protein can have profound consequences upon its function in cells. Given that p53 is known to be responsive to a variety of stress signals, considerable attention has focused on the determination of phosphorylation sites within the N-terminal activation region and the C-terminal regulatory domain (Figure 1). A number of human p53 sites have been identified that are known to be phosphorylated in vivo, and several kinases have been identified that can phosphorylate these same sites in vitro. However, evidence is only indirect at present for utilization of some of the N-terminal sites.

One key technical breakthrough in determining stress-induced changes in phosphorylation has been the use of phosphorylation site-specific antibodies. Such reagents have enabled the detection of specific constitutive or dynamic phosphorylation events with a sensitivity and rapidity that was previously impossible. Using such an antibody, Ser15 was the first human p53 site shown to be inducibly phosphorylated after DNA damage (Shieh et al., 1997; Siliciano et al., 1997). This site is a known

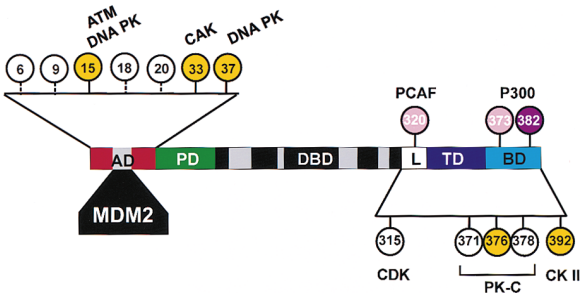

Figure 1. Stress-Induced Modifications of Human p53 Protein

Domains or regions of p53 indicated are as follows: activation domain (AD), residues 1–60; growth suppression PXXP region (PD), residues 63–97; DNA-binding domain (DBD), residues 100–300; linker region (L), residues 305–323; tetramerization domain (TD), residues 323–356; and basic C-terminal regulatory region (BD), residues 363–393. Sites at N terminus include potential sites (Ser6, -9 and -20, and Thr18) and sites known to be phosphorylated in vitro by ATM, DNA-PK, or the cyclin-activating kinase complex, CAK, as indicated. C-terminal phosphorylation sites for CDK, PK-C and CKII protein kinases, and PCAF and p300 acetyl transferases are indicated. Altered modification sites after DNA damage are shown in yellow for phosphorylation sites and purple for acetylation site. Gray regions indicate highly conserved regions on p53, including region I, which interacts with MDM2.

substrate for the DNA-activated protein kinase (DNA-PK). Although it is unclear whether DNA-PK phosphorylates p53 directly in vivo, the recent observation of Woo et al. (1998) showing that p53 is inert as a transcriptional activator in cells lacking detectable DNA-PK activity suggests that indeed DNA-PK is upstream of p53 and is required for its transcription function. Ser15 has also been shown to be phosphorylated by another kinase, the product of the *ATM* gene (Banin et al., 1998; Canman et al., 1998), that is defective in patients with ataxia telangiectasia (AT), a syndrome characterized by pleiotropic phenotypes including extreme sensitivity to ionizing radiation. Importantly, ATM kinase activity is increased after DNA damage, providing a tantalizing hint that ATM may phosphorylate p53 in vivo. Phosphorylation of Ser15 is delayed in cells from AT patients (AT^- cells) after gamma irradiation (IR) but not UV, suggesting that ATM is important for signaling to p53, but in a DNA damage–specific manner (Siliciano et al., 1997). Nevertheless, in AT^- cells p53 does eventually become phosphorylated at Ser15 after IR, indicating either that other kinases can substitute for ATM or that the kinetics of the bona fide primary Ser15 kinase (as yet unidentified) is regulated by ATM kinase.

That Ser15 is a target for different protein kinases suggests that modification of this site is important for altering p53 after DNA damage. Indeed, Shieh et al. (1997) found that DNA damage-induced phosphorylation at Ser15, which lies at the N-terminal border of the MDM2 interaction region, weakens both the p53 association with MDM2 and the ensuing ability of MDM2 to repress transcriptional activation by p53. This observation provides a paradigm for how DNA damage–induced phosphorylation might lead to stabilization and activation of p53. Additional sites within the N terminus are likely to be inducibly phosphorylated (Siliciano et al., 1997; Sakaguchi et al., 1998; see Figure 1), and it will be important to continue to explore how N-terminal phosphorylation and kinases affect p53 stability and interaction with MDM2.

Stress-induced changes are not relegated solely to the N terminus of p53, and several recent studies have reported alterations in C-terminal modification sites. Two groups reported that UV but not IR induces phosphorylation of murine Ser389 (Kapoor and Lozano, 1998; Lu et al., 1998). Thus, as is the case with ATM, different types of DNA damage are relayed through discrete pathways to p53. Not only kinases are signaling targets: Waterman et al. (1998) made the interesting observation that Ser376, previously shown to be a protein kinase-C (PK-C) site, is actually dephosphorylated after IR in normal but not AT^- cells, implying that a stress-responsive phosphatase may also regulate p53 modification. Loss of phosphate at this residue is correlated with both binding of p53 to 14-3-3 proteins and activation of sequence-specific DNA binding. Furthermore, DNA damage-inducible acetylation of a C-terminal residue (Lys382) was recently reported (Sakaguchi et al., 1998), showing that signaling is not exclusive to changes in phosphorylation.

How might these C-terminal modifications affect p53's function? As mentioned above, it has been known for some time that p53 can be isolated in a form that is virtually incapable of binding to DNA, due to the fact that the highly basic p53 C terminus can negatively regulate the central sequence-specific DNA-binding region. Phosphorylation of either cyclin-dependent kinase (CDK), PK-C, or casein kinase II (CKII) sites within this region relieves autorepression of the central domain by the C terminus. More recently it was also reported that acetylation of p53 at C-terminal lysine residues accomplishes a similar result (Gu and Roeder, 1997; Sakaguchi et al., 1998). Both phosphorylation and acetylation are likely to work by counteracting the effects of the highly positively charged C terminus. However, noncovalent modifiers such as antibodies, short single strands of DNA, or the redox-repair protein, REF1, can also activate latent p53. Since these experiments have for the most part been performed in vitro, it has been gratifying to identify altered modifications of p53 in vivo after stress signals that might lead to its activation. The fact that the C terminus is subjected to multiple changes after DNA damage lends credence to the concept that p53 can exist in a latent state until it is needed to function as a regulator of cell growth or death. Whether any given modification results in selective activation of different p53 functions or outcomes will be of considerable interest in the future. Furthermore, it will be important to determine whether changes in p53 conformation due to C-terminal modification will be propagated to the N-terminal region and thus affect its interaction with MDM2.

The Discovery of ARF Explains How Oncogenes Regulate p53

How p53 becomes stabilized by viral and cellular oncogenes remained a complete mystery until quite recently. The discovery of the product of an ARF within the *p16^{INK4a}* locus encoding an inhibitor of cyclin D–associated CDKs has led to a flurry of studies that have provided a quantum leap in our understanding of how p53 stabilization might occur. Murine *ARF*-null mice are highly tumor

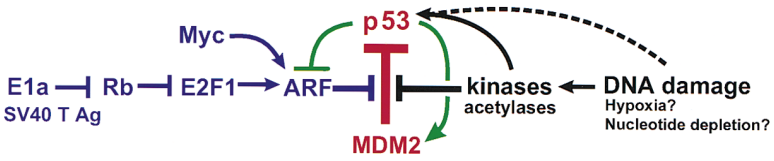

Figure 2. Oncogenes and Stress Signals Independently Activate p53 through MDM2

prone, and their mouse embryo fibroblasts, like those lacking p53, are immortal (Kamijo et al., 1997). Overexpression of $p19^{ARF}$ in wild-type but not $p53^{-/-}$ cells causes cell cycle arrest, suggesting that ARF acts upstream of p53 (Kamijo et al., 1998). Moreover, in wild-type cells ARF overexpression leads to increased levels of p53 and the p53 target gene, *p21* (Kamijo et al., 1998). Strikingly, however, in $ARF^{-/-}$ cells p53 induction and a p53 cellular response to DNA damage is intact (Kamijo et al., 1997). Thus, under some conditions ARF is required to induce p53, but its regulation of p53 is separate and independent from DNA damage–induced signaling to the p53 protein.

How does ARF induce p53? A number of studies have revealed direct physical interactions between ARF, p53, and MDM2. Several groups (Kamijo et al. 1998; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998) have found that ARF binds to MDM2 and that trimeric complexes between p53, MDM2, and ARF can form in vivo. The N-terminal 62 amino acids of murine ARF are sufficient for it to bind to the C-terminus of MDM2 within a region not required for MDM2 association with p53 (Kamijo et al. 1998; Zhang et al., 1998). However, conclusions about the consequences of these interactions differ among the different investigators: Zhang et al. (1998) reported that ARF can itself target MDM2 for degradation. The other studies, however, do not suggest this as a mechanism for how the association of ARF with MDM2 leads to increased quantities of p53, and more work will be required to reveal the mode(s) of action of ARF on MDM2 and p53.

With the excitement following the discovery that ARF regulates p53, researchers were keen to unearth how ARF is itself triggered in cells. It is now clear that ARF is induced by viral and cellular oncogenes, thereby countering hyperproliferative signals by inducing p53-dependent apoptosis. We can envisage a cellular pathway in which ARF levels are regulated by the retinoblastoma protein, Rb, another well-studied tumor suppressor (Figure 2). Normally, hypophosphorylated Rb binds and blocks the activity of members of the E2F family of transcription factors, including E2F1. The E2Fs function to activate a number of genes required for passage into and through S phase. Bates et al. (1998) now report that E2F1 induces ARF expression most likely through its ability to activate the ARF promoter. The findings in two current papers using mouse models, that E2F1 is instrumental in regulating p53-mediated apoptosis (Pan et al., 1998; Tsai et al., 1998), fit nicely with this observation.

DNA tumor virus products can associate with Rb, disrupting its ability to block passage of cells through G1 phase. It has now been shown that E1a induces ARF expression in cells, and importantly, the ability of E1a to stabilize p53 is absent in $ARF^{-/-}$ fibroblasts (de Stanchina et al., 1998). Indeed, the E1a result beautifully connects the Rb pathway to p53: by counteracting the ability of Rb to repress E2F, E1a promotes ARF activation and consequent p53 stabilization. Consistent with the observation that the p53 DNA damage–responsive pathway is intact in $ARF^{-/-}$ cells, de Stanchina et al. (1998) have found that p53 phosphorylation on Ser15 occurs in DNA-damaged, but not E1A-expressing, cells.

Not only viral but also cellular oncogenes can regulate p53 through ARF. Zindy et al. (1998) have demonstrated that Myc, presumably working through an independent mechanism, can induce ARF. Intriguingly, Myc overexpression in mouse embryo fibroblasts rapidly leads to the appearance of variants that lose either ARF or p53 function giving rise to immortal cells that are resistant to Myc-induced apoptosis and that can be transformed by oncogenic *Ras* alleles alone (Zindy et al., 1998). It is interesting to speculate that immortalization of primary fibroblasts by genes like *E1a* and *Myc* and their cotransformation by *Ras* may depend in part on the ability of Myc and E1a to enforce selection of cells that have dismantled the ARF–p53 checkpoint. This conjecture is supported by the observation that overexpression of $p19^{ARF}$ counteracts the ability of Myc and Ras to transform wild-type but not $p53^{-/-}$ cells (Pomerantz et al. 1998). It is anticipated that yet additional pathways will connect oncogenes to p53 through ARF, and a recent report from Palmero et al. (1998) suggests that Ras may also be involved in this process.

The Complexity of p53 Circuitry

The studies described above are but another chapter in the continuing saga of deciphering p53. We now have further evidence that p53, a key cellular regulator, is under the control of MDM2. But intricate circuitry is at play here: p53 itself is instrumental in upregulating its inhibitor, MDM2, and also downregulating its activator, ARF, as depicted in Figure 2. It is well established that MDM2 is itself a transcriptional target of p53 that is induced after p53 becomes stabilized and activated. There is now evidence that p53 downregulates ARF under normal conditions (Stott et al., 1998). Thus, by interacting with MDM2 and inhibiting ARF expression, p53 levels are kept low during normal conditions. After stress, modification of the p53 protein prevents or disrupts the p53–MDM2 interaction, while as a result of oncogene imbalance, ARF is induced and MDM2 is prevented from destabilizing p53. The outcome in both cases, though, is increased and activated p53 protein. Overlaid on the p53–MDM2 binary switch and its regulators is circuitry connecting the two tumor suppressors, p53 and Rb. Activation of p53 after DNA damage leads to G1 arrest that, through p21, occurs by maintenance of active unphosphorylated Rb, which in turn restrains E2Fs. Thus, in this situation p53 can be seen as upstream of Rb. E1a and T antigen can bind to and inactivate Rb, and both can stabilize p53 through the ARF

pathway. Here the case is reversed and now Rb becomes the regulator of p53. The fact that the *INK4A* locus, frequently deleted in tumors, encodes overlapping regulators of Rb and p53, further attests to the intimate relationship between these two tumor suppressors, long suspected by DNA tumor virologists to be intertwined.

Future Directions

As is often the case with new discoveries, new questions are posed: What are the protein kinases involved in phosphorylating p53 sites that regulate its interactions with MDM2? Do different forms of genotoxic stress activate discrete upstream kinase signaling cascades and result in different outcomes, such as apoptosis versus cell cycle arrest? How does phosphorylation of N-terminal sites affect p53's interaction not only with MDM2, but also with transcription factors and with its DNA sites? How do Myc and Ras signal to ARF? The model presented in Figure 2, suggesting that release of E2F is sufficient to stabilize p53, is likely to be an oversimplification. Since E2F activity is liberated during transit to S phase, how is p53 checkpoint function reigned in during normal passage of cells through the cycle? Oncogenes may not only work to relieve repression of E2F; perhaps they can also prevent downregulation of ARF by p53. Are there signaling and stabilization pathways to p53 that are separate from the two outlined in Figure 2? How does p53 get back to ground state once stabilized by either ARF or DNA damage? And perhaps the most important question of all: can these discoveries be used for therapeutic purposes? Answers to these and related questions are likely to keep many of us busy for the forseeable future.

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