

MINIREVIEW

Functional Regulative Pathways for p53, a Protein of Basic Importance for the Integrity of the Cell Genome

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The aims of this commentary are to underline how complex is the functional regulation of p53 and to describe the distinct molecular pathways that control two fundamental functions of p53: surveillance of genome integrity and apoptosis. We shall consider the following: regulation of the p53 half-life, the MDM2 gene feedback loop, p53 nuclearization, the balance between active and latent p53 isoforms, p53 alternative splicing, p53 acetylation at the carboxyl terminal, regulation of the p53 central binding domain, and the p53 relationship with tumor promoters.

p53 is a transcription factor that activates genes involved in key events of cell life such as those regulating cell cycle checkpoints, DNA repair, cell growth, differentiation, apoptosis, and senescence (1). p53 controls genome integrity and the proper balance between growth and apoptosis, thus leading Lane (2) to define it as “the guardian of the genome.” This famous definition is strengthened by the observation that p53 is lost or inactivated in more than 60% of human malignant cancers (3). This important protein responsible for genome integrity has to be tightly regulated in normally growing cells; thus, p53 is almost absent during normal cell proliferation, exhibiting a very short half-life (4). DNA double-strand breaks caused by ionizing radiations or by DNA-damaging agents induce a significant increase of p53. Then it migrates into the nucleus and it halts the cell cycle at G1/S by inducing the expression of the cell cycle inhibitor WAF1; in addition to G1/S, p53 also works at the G2/M checkpoint (5–7). Thus, the cell gains time to repair DNA or, if impos-

sible, to enter apoptosis by down-regulating the anti-apoptotic gene bcl-2 and up-regulating the pro-apoptotic gene bax (8). At this point, the so-called caspase cascade is activated and caspases, a family of proteases, destroys proteins that are essential for cell life (9). However, in apoptosis non-proficient cells (i.e., diploid human fibroblasts), p53 induces a prolonged G0/G1 arrest, thus causing cellular senescence by the transcription of the WAF1 gene homolog termed senescence-derived inhibitor-1 (SDI-1) (10,11).

p53 is very finely tuned: A redundant number of regulative pathways often are simultaneously at work, as a kind of insurance that at least one of them is active under different conditions.

PROTEOLYTIC DEGRADATIONS BY CALPAIN AND THE UBIQUITIN-PROTEASOME COMPLEX

Calpain and ubiquitin are two intracellular proteases that degrade p53; however, it is not known whether these proteases cleave p53 when it is located in the cytoplasm or in the nucleus. There exist two isoforms of calpain that differ in their calcium requirements (either millimolar or micromolar); thus, the activity of calpain, a cysteine protease, is dependent on intracellular calcium mobilization and on the presence of PEST (P, proline; E, glutamic or aspartic acid; S, serine; and T, threonine) sequences on the target protein. Indeed, p53 has a strong PEST sequence that is likely to be important for calpain susceptibility. Oddly enough, mutated p53 isoforms

featuring a prolonged half-life do not show PEST sequences but are still susceptible to calpain degradation (12). It seems that p53 protein stability is more dependent on nuclearization rather than on reduced proteolysis and, consistently, most mutated p53 proteins have a strong tendency to nuclearization. In any case, the increase of the p53 half-life is remarkable: from minutes to hours and even days (13).

The main difference between ubiquitin- and calpain-dependent p53 degradation is the calcium requirement in that ubiquitin does not require an increased calcium concentration to cleave p53. However, calpain requirement of calcium suggests that the cell calcium homeostatic mechanisms, such as the voltage operating calcium channels (VOCs), the receptor operating calcium channels (ROCs), the calcium exchangers, the energy-dependent pumps, and the inositol lipid metabolism are critical for regulating calpain activity. These calcium-increasing mechanisms are normally activated following growth factor-dependent stimulation of cell proliferation and cell cycle entry. Thus, calpain-dependent p53 degradation during normal cell growth is consistent with the need to get rid of p53, a factor that normally inhibits cell proliferation (8).

Maki *et al.* (14) provided evidence for *in vivo* ubiquitination and 26S proteasome-mediated degradation of p53 in various human diploid cell lines. This phenomenon appears to be common to many cell lines such as RKO, SAOS-2, and U2OS (human osteosarcoma). The E6 protein of the cancer-associated virus HPV16/18, in cooperation with a cellular factor termed E6AP, forms a complex with p53 *in vivo*, thus promoting its ubiquitination and subsequent degradation by the proteasome pathway. However, ubiquitin is able to degrade p53 even in the absence of the viral factor (14). Ubiquitin-dependent proteolysis plays an important role in the degradation of short-lived regulatory proteins such as p53. Three enzymatic activities are involved in the ubiquitination of a protein: Ubiquitin is first activated through its covalent thioester linkage to the E1 ubiquitin-activating enzyme; then, in the form of a high-energy thioester bond, ubiquitin is transferred to the E2 enzyme. E2 is able to link ubiquitin to the substrate or, alternatively, transfer it to E3 ligase, which performs the ubiquitination. Either in the direct or in the indirect ubiquitin transfer, E3 is involved in recognition of the target substrate by E2 that directs the hydrolytic activity of the proteasome (15). However, the factor(s) or cellular conditions

stimulating ubiquitin-dependent degradation of p53 are still unknown; neither is it known whether mutated p53 is resistant to this type of degradation. It could be suggested that, unlike calpain, whose degradative activity might be regulated by proliferation, ubiquitin degradation of p53 is more likely a constitutive pathway responsible for the short half-life of p53 protein.

MDM2-RELATED INACTIVATION OF p53

Mouse Double Minute 2 (MDM2) is a gene, first discovered in mouse, that in humans codes for a 491-amino-acid protein which binds p53 at the amino-terminal transcriptional domain. It is proposable that this event might inhibit the transcriptional activity of p53 on target genes, thus inducing a loss of p53 activity. The interval between p53 activation and MDM2 accumulation defines a time window during which p53 exerts its effects (16). Accordingly, artificial overexpression of MDM2 strongly inhibits p53 accumulation (17). However, in spontaneous human tumors, the primary p53 loss of function does not allow the expression of MDM2, and this could be one of the causes that leads to p53 accumulation in cancer. Thus, it has been proposed that MDM2 overexpression leads to experimental cancer because it prevents the protective function performed by p53 on genome integrity (18).

NUCLEARIZATION

Treatment of normal fibroblasts with DNA-damaging agents (such as cancer therapy drugs or radiations) induces nuclear accumulation of p53 (19). p53 nuclear accumulation begins few hours after treatment and remains detectable in surviving cells for at least 20 days. Accumulation occurs because of increased protein stability and it is dependent on ongoing translation. A number of cell cycle inhibitors (such mimosine, afidicoline, or nocodazole) does not affect nuclearization, thus suggesting that this process may begin from several points of the cell cycle. These observations are in agreement with the hypothesis that DNA damage itself induces p53 nuclear accumulation: this event leads either to inhibition of cell growth at definite checkpoints by expression of WAF1, allowing time for DNA repair, or, in the case of severe damage, to apoptosis. Surviving cells that escape apoptosis enter a growth arrest period at the G0/G1 transition and, after some weeks, they enlarge, assuming an irregular shape

similar to that of senescent cells. It has been proposed that in this case cells enter a slow death process resembling that of senescence (11). If this hypothesis is true, then p53 may have three different functions in DNA-damaged cells: cell cycle arrest for DNA repair, cell death by apoptosis, and slow death by senescence. The major nuclear localization signal (NLS) of p53 is a highly conserved amino acid sequence located near its carboxyl terminal. The NLS is required to target the p53 protein into the nucleus, but the complex molecular mechanisms responsible for the nuclear shift from the cytoplasm are still quite obscure. Likewise, it is not fully understood why p53 stability increases from minutes to weeks as result of its shift to the nucleus. Most likely more than one factor is involved and it is possible that DNA bound p53 is either more stable or more protected from degradation. Thus, Molinari and Milner (20) showed that p53 is resistant to ubiquitin-dependent proteolysis when it is complexed with DNA. It appears that the stockpiling of p53 in the nucleus is due to increased protein stability and/or an increased translation, since no change in p53 mRNA level was observed.

ACTIVE AND LATENT ISOFORMS

Key elements of cell physiology are often regulated in a complex and redundant manner. In addition to half-life regulation by DNA binding and MDM2-related inactivation, the activity of p53 is also regulated by the balance between active and inactive (or latent) isoforms. This balance is not constant and may change in distinct occurrences such as cell growth stimulation, DNA damage, cell differentiation, and apoptosis. Hupp *et al.* (21) propose an allosteric model to explain the molecular differences as well as the possibility of reversal of the latent and active p53 isoforms. The key elements of this model are the carboxyl terminals (“tails”) of the p53 tetramer that interact with the core of the molecule. This interaction locks the core in a conformation that is inactive because cannot bind to specific DNA consensus motifs. When this “tail–core” interaction is disrupted by covalent modifications, or by mutations affecting the carboxyl terminal of p53, the core activity of DNA binding is restored. DNA damage caused by irradiation, p53 phosphorylation by some kinases, removal of negative regulatory domains by alternative splicing, binding to Hsp70 heat-shock protein, competitive binding of artificial peptides to the carboxyl terminal, and binding to the antibody

Pab421 (22) are among the mechanisms that activate the p53 tetramer from latent to active. It is worth noting that all these events converge toward carboxyl-terminal modifications. Although some of the above mentioned occurrences are experimental in nature, phosphorylation and alternative splicing are physiological events, but unfortunately their meaning is still poorly understood. A hypothesis that might help elucidating why p53 is organized in a latent isoform is that the cell has a pool of p53 molecules ready to be used in response to cell stress signals and that could be rapidly activated by simple phosphorylation at the carboxyl terminal; this phosphorylation could be carried out by signal-dependent kinases such as protein kinase C (PKC).

ACETYLATION OF THE CARBOXYL TERMINAL

Gu and Roeder (23), proposed that acetylation of lysine residues at the carboxyl terminal of p53 protein is a novel mechanism for its functional regulation. These authors demonstrated that p53 acetylation could occur both *in vitro* and *in vivo*. Remarkably, the site of p53 that is acetylated by its cofactor, CBP/p300 (24) is located inside the carboxyl terminal domain itself, further indicating that this region of p53 protein might be critical for non-specific DNA binding. Acetylation of the carboxyl terminal domain could dramatically stimulate the sequence-specific DNA binding activity of the core domain, possibly as result of acetylation-driven conformational change. These observations suggest another pathway for p53 regulation and provide an example of acetylation-mediated modulation of a specific protein activity. Thus, it is well known that histone acetylation modifying the chromatin structure leads to activation of gene transcription. Unfortunately, which is the exact domain of cellular p53 protein that is acetylated in normal cells, and how is p53 acetylation regulated—particularly in response to specific stimuli (e.g., DNA damage)—still remains unknown. Since the cancer therapy drug etoposide is able to activate p53, switching it from the latent to the active form (25), it should be very interesting to study whether this activation is related to p53 protein acetylation in response to drug-induced DNA damage, as is the case of carboxyl terminal phosphorylation. In other words, it would be interesting to study whether acetylation of p53 protein is an ancillary or a relevant pathway for its activation in response to DNA damage.

ALTERNATIVE SPLICING AT THE CARBOXYL TERMINAL

More than 10 years ago, Arai *et al.* (26) discovered the existence of an alternative splicing for p53 mRNA, generating two distinct proteins. The alternative splicing occurs by insertion of 96 nucleotides in the 3' end of intron 10, between nucleotides 1091 and 1092 of the p53 mRNA: Translation of the alternatively spliced molecule results in the truncation of 9 amino acids and in the substitution of 17 amino acids at the carboxyl terminal. Han and Kulesz-Martin (27) showed that the protein coded for by the alternatively spliced mRNA, named p53AS (alternatively spliced), accounts for about 30% of the total cellular p53. p53AS is preferentially expressed during the G2 phase of the cell cycle, where it might exert some still unknown function. Wu *et al.* (28) showed that p53NS (i.e., normally spliced) was able to promote the reassociation of single-strand DNA into duplex molecules; on the contrary, p53AS completely inhibited the formation of DNA/DNA double strands: These data were the first indication that p53AS might feature distinct functional properties.

Since it is well documented that chromosome integrity at the G2/M transition is protected by p53 that prevents gene amplification, aneuploidy, and other gross chromosomal aberrations (29), it is proposable that the p53 protein acting at the G2/M transition is just p53AS: Thus, at this checkpoint p53 could operate by avoiding stickiness of single DNA strands emerging as a consequence of DNA damage. Furthermore, p53 working at the G2/M transition might inhibit aberrant reannealing that is responsible for asymmetric crossing-over, leading to gene amplification and other gross chromosomal aberrations.

Although speculative at present, these observations about the hypothetical activity of p53AS at G2/M, further point to the carboxyl terminal of the protein as a critical domain for regulating its function. As a word of caution, it should be considered that p53AS, so far, has been found only in mice.

CARBOXYL TERMINAL REGULATION OF THE SPECIFIC DNA-BINDING DOMAIN

The different domains of human p53 protein are well characterized: A 42-amino-acid amino terminal domain with specific transcriptional activity positively regulating target gene expression; a sequence-

specific DNA-binding core domain, interacting with a consensus DNA sequence located in the center of the molecule (from aminoacid residues 102 and 292), where mutations often occur; a carboxyl-terminal region from amino acid 324 to the last residue (No. 393) which includes nuclearization and tetramerization domains and is involved in the nonspecific re-association of nucleic acid strands and in the inhibitory control of the sequence-specific DNA-binding site. When p53 carboxyl terminals are nonspecifically bound to DNA, they prevent recognition of the specific DNA consensus sequence, thus precluding the transcription of genes that are under the control of p53 (30). The nonspecific DNA-binding activity of the carboxyl terminal locks the whole tetramer in the inactive state. Consequently, p53 with carboxyl terminals in the "nonfunctioning mode" is a latent, inactive molecule, although it is ready to be promptly switched on by different mechanisms.

A brief review of the molecular mechanisms acting on p53 carboxyl-terminal regulation could start with *in vitro* experiments performed with purified baculovirus-expressed p53: It is well known that binding of the antibody Pab421 to the epitope 370–378 activates p53 (22). The same is true for peptides or oligonucleotides that bind to the carboxyl terminal (21). Activation of p53 also occurs following deletion of the carboxyl-terminal itself (28). All these experimental evidences might not reflect exactly what occurs *in vivo*, but they provide important indications of the molecular mechanisms regulating the shift from a latent to an active molecule. There are at least four models for the p53 activation, those proposed by Hupp *et al.* (21), Bayle *et al.* (30), Gu and Reader (23), and Anderson *et al.* (31). However, it is difficult to predict which one, if any, of these hypothesized mechanisms is actually operating *in vivo*.

The model proposed by Hupp *et al.* (21) has been previously described, and it is based on a core–tail interaction that locks the molecule in the inactive state. The critical point of this model is the demonstration of the tail–core interaction; furthermore, this model is not in agreement with the finding that carboxyl terminals have a nonspecific DNA-binding activity that prevents p53-specific binding to DNA.

Gu *et al.* (24) found that when p53 is able to specifically bind DNA, the carboxyl terminals are acetylated. The model they propose is very similar to that of Hupp *et al.* (21): Full-length p53 exists in a latent form as a result of the tail–core interaction;

acetylation of the lysine residues of the carboxyl-terminal regions results in neutralization of positive charges and leads to disruption of the interaction between the carboxyl-terminal domain and the core domain, a mechanism resembling that occurring with phosphorylation.

A different model for the regulation of sequence-specific DNA binding by p53 carboxyl terminals was proposed by Bayle *et al.* (30). The most notable point of this model is the following: the central DNA-binding domain of p53 is responsible for the specific interaction with the consensus motifs. However, its activity is impaired by nonspecific binding of the carboxyl terminals to cellular DNA. p53AS, because of amino acids deletion, lacks the nonspecific DNA-binding elements, while it still maintains the ability to interact with the specific recognition element. Thus, the antibody Pab421 inhibits the nonspecific DNA-binding activity, activating in this way p53. Following DNA damage, double-strand alterations are recognized by p53 carboxyl terminals. A mechanism through which p53 protein may be activated for sequence-specific DNA-binding could imply disengagement of the carboxyl terminals involved in the nonspecific DNA binding through phosphorylation by co-localized protein kinases present at the site of DNA lesions. Thus, this covalent modification would interrupt the nonspecific interaction between the carboxyl terminal and DNA, allowing p53 to specifically induce the transcriptions of genes under its control. It appears that the function of the carboxyl terminals (as proposed by the Bayle *et al.* (30)) is mainly concerned with DNA damage recognition and p53 activation, especially so when phosphorylation occurs. It is proposable that in this manner cells may count on a considerable pool of latent p53 molecules, nonspecifically bound to DNA by their tails. These latent p53 proteins would not interfere with physiological growth, but they would be ready to bind DNA through their core domain when DNA damage-dependent kinases unfasten p53 tails. In this respect, the model proposed Anderson *et al.* (31) does not significantly differ from that proposed Bayle *et al.* (30).

The emerging picture is very complex and the presence of many different models demonstrates that the understanding of the actual role of the carboxyl terminals in p53 functional regulation requires further studies.

PHOSPHORYLATIVE REGULATION OF p53 AND ITS RELATIONSHIPS WITH TUMOR PROMOTERS

Two biochemical events appear to be often involved in the functional modification of p53 activity: mutations of the gene coding for p53 or phosphorylation/dephosphorylation of p53 protein. These events bear completely different implications: Mutations are stable genetic alterations transmitted to the cell progeny and may influence p53 functions with opposite results: loss of functions and dominant negative effects, or dominant positive transforming effects, associated with gain of function (for review, see Refs. (32) and (33)). On the other hand, phosphorylation/dephosphorylation is a mechanism responsible for transient, functional activation/inactivation of p53 protein activity; this mechanism is at work in different occurrences such as DNA damage, cell signaling, and cell cycling. The wide variety of kinases involved in p53 phosphorylation might underline the importance of this event in the p53-dependent regulation of cell growth. So far eight different kinases involved in p53 phosphorylation have been described (for review, see Ref. (34)); however, despite the kinase abundance, the exact role of this event in the regulation of p53 activity during cell proliferation is still largely unknown.

Phosphorylation of the carboxyl terminal (epitope from amino acid 370 to 378) carried out by PKC bears consequences similar to those of acetylation of the same epitope; thus, steric exclusion of the carboxyl terminal appears to be a necessary event to activate the latent p53 tetramer to bind specific DNA consensus sequences. The phosphate groups covalently bound to p53 by PKC would be instrumental in preventing carboxyl terminal nonspecific binding to DNA. Thus, negative phosphate groups attached to the p53 carboxyl terminal have a repulsive effect on DNA phosphates. However, according to an observation by Milne *et al.* (35), phosphorylation of p53 is not accomplished by PCK alone, but as part of the mitosis-associated protein kinase cascade.

Delphin and Baudier (36) demonstrated that short-term treatment with exogenously added PKC agonists such as tumor-promoting phorbol esters causes PKC-dependent phosphorylation of p53 and its consequent specific binding to DNA. However, it is well assessed that, when the administration of phorbol esters is prolonged, such as in "chronic treatment," some PKC isoforms are down-regulated

(37,38). In our laboratory we studied p53-null K562 human leukemia cells transfected with a temperature-sensitive mutant of p53; these cells are able to undergo p53-dependent apoptosis at the permissive temperature, possibly because they have gross genetic alterations typical of neoplastic cells. We observed that p53-expressing K562 cells, when at the permissive temperature, are protected from apoptosis by chronic phorbol ester treatment, thus suggesting that p53 might be in an inactive form following "chronic" PKC down-regulation (39).

It could be proposed that chronic treatment with tumor promoters, as in the case of experimental mouse skin tumors, induces PKC down-regulation with a consequent decrease of p53 phosphorylation, this favoring the maintenance of p53 in a latent form. Consistent with this interpretation, Kemp *et al.* (40) showed that carcinogenesis by phorbol esters is greatly enhanced when p53 loses its function. Endogenously produced PKC agonists, like the mitogenic second messenger diacylglycerol (DAG), act like tumor promoters and are as efficient as phorbol esters in experimental mouse skin carcinogenesis (41,42). In spontaneous human cancer, it was demonstrated that the level of DAG is significantly increased in malignant lung cancer cells compared to normal cells (43). This DAG increase could be related to transformation by dominant oncogenes, like *src* and *ras*, which, under experimental conditions, induce a sustained *de novo* synthesis of DAG through the glycolytic pathway (44). Constitutive production of DAG, as observed in oncogene-transformed cells and in human cancer, causes a significant down-regulation of PKC that might in turn influence the status of p53 phosphorylation (45).

CONCLUSIONS

Rather than a guardian, p53 could be considered a ferocious watch dog of vital cell function: If it senses a danger, it is ready to kill rather than allowing a crime against the integrity of the genome. Such a watch dog has to be kept on a short leash to prevent unwanted bites against normally growing cells; thus, the complex and redundant functional regulation of p53. Actually, more than a single leash, there are several restraints that control p53 activity and, accordingly, there are several escape mechanisms that allow p53 to exert its function of protecting the genome.

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