Biogenesis of p53 Involves Cotranslational Dimerization of Monomers and Posttranslational Dimerization of Dimers

IMPLICATIONS ON THE DOMINANT NEGATIVE EFFECT*

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Precisely how mutant p53 exerts a dominant negative effect over wild type p53 has been an enigma. To understand how wild type and mutant p53 form hetero-oligomers, we studied p53 biogenesis in vitro. We show here that p53 dimers are formed cotranslationally (on the polysome), whereas tetramers are formed posttranslationally (by the dimerization of dimers in solution). Coexpression of wild type and mutant p53 therefore results in 50% of the p53 generated being heterotetramers comprised of a single species: wild type dimer/mutant dimer. Using hot spot mutants of p53 and a variety of natural target sites, we show that all wild type/mutant heterotetramers manifest impaired DNA binding activity. This impairment is not due to the mutant dimeric subunit inhibiting association of the complex with DNA but rather due to the lack of significant contribution (positive cooperativity) from the mutant partner. For all heterotetramers, bias in binding is particularly pronounced against those sequences in genes responsible for apoptosis rather than cell growth arrest. These results explain the molecular basis of p53 dominant negative effect and suggest a functional role in the regulation of p53 tetramerization.

The importance of p53 as a tumor suppressor has been well documented. Over half of all human cancers are mutated in the gene encoding p53, and many viruses can induce transformation of the host cell through p53 inactivation (Ref. 1; reviewed in Refs. 2 and 3). Furthermore, inheriting a germline p53 mutation characteristic of Li-Fraumeni syndrome confers a strong predisposition to cancer because 50% of those afflicted acquire cancer by age 30 (4, 5). The role of p53 in tumorigenesis was also demonstrated in an animal tumor model, in which p53−/− mice were found to be more prone to cancer development (primarily lymphomas) compared with their wild type (p53+/−) and heterozygous (p53+/−) littermates (6, 7). Loss of p53 or its function is therefore clearly linked to tumor formation.

As “guardian of the genome,” p53 is activated by a number of genotoxic and stress signals such as ionizing radiation, ultraviolet light, ribonucleotide depletion, hypoxia, oxidative stress, heat shock, and exposure to nitric oxide (reviewed in Ref. 8). Critical to the tumor-suppressing function of activated p53 is its ability to bind sequence-specific DNA sites and induce the transcription of genes involved in cell cycle arrest, DNA repair, and apoptosis. This is illustrated by the fact that most p53 mutations occur in its DNA-binding domain and affect its sequence-specific DNA binding ability (1). Such mutations (called “hot spot” mutations) can be divided into two categories; class I mutations affect residues that make direct contact with DNA, whereas class II mutations occur at residues crucial for maintaining the conformation of the DNA-binding domain (9).

The consensus p53 binding site consists of two or more copies of the 10-bp half-site 5′-PuPuPuClA/T(A/T)GPyPyPy-3′ separated by up to 13 bp (10). Minor variations to this consensus sequence are found in all p53 target genes. Each half-site consists of two inverted repeat 5-bp quarter sites. p53 binds to this consensus DNA sequence as a pair of clamps, with the two monomers within each dimer binding to two consecutive quarter sites within a half-site (11). The two dimers within the tetramer therefore bind to the two half-sites in juxtaposition to each other, resulting in overall enhanced stability of the p53-DNA complex. The lack of such cooperative binding in a single dimer-half-site interaction accounts for the drastically reduced stability of the dimer-half-site complex relative to that of the tetramer-full site complex. This is also reflected by the observation that in contrast to full sites, half-sites do not confer transcriptional responsiveness to p53.

Although there is little doubt that tumors often arise through deletion or mutation of both p53 alleles (the two-hit model) (12), there is increasing evidence that a point mutation or deletion in a single allele could result in increased susceptibility to cancer (13–17). In cases where both mutant and wild type p53 are expressed, it has been suggested that the mutant protein exerts a dominant negative effect over the wild type protein, essentially rendering the latter inactive (reviewed in Refs. 18 and 19). The end result is a drastic decrease in the level of functional p53, which in turn promotes genomic instability and cancer development. That the mere reduction in functional p53 levels may be sufficient to promote tumorigenesis has also been recently shown in an animal tumor model in which heterozygous p53+/− mice containing a single wild type p53 allele develop tumors much earlier than those mice with two functional p53 alleles (17).

The fact that many human tumors contain both a mutant and a wild type allele has led to speculations as to how mutant transmission of this article was defrayed in part by the payment of page charges. This article must therefore be hereby marked “in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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p53 can affect wild type p53 function. It has been proposed (and generally believed) that in such tumors, the mutant p53 protein complexes with the wild type counterpart and drives the latter into a mutant conformation that is nonfunctional (i.e., incapable of binding DNA). This model portrays mutant p53 as being dominant over wild type p53, leading to the so-called “dominant negative” effect. Support for this model has come largely from experiments in which mutant and wild type p53 were found to be coprecipitatable when they are coexpressed (20). More recent data suggest that the C-terminal oligomerization domain of p53 is absolutely required for the manifestation of this dominant negative effect, because p53 mutants without a functional tetramerization domain are not dominant negative and not oncogenic (21, 22). Although it now seems clear that p53 mutants need to interact with wild type p53 to impart a dominant negative effect (23), there is no concrete evidence that this effect is the result of an induced conformational change of the wild type protein. Indeed, it has been reported that mutant p53 proteins that have a wild type conformation (class I mutants) are also able to impart a dominant negative effect (24). In corroboration with the above observation was the demonstration that the C-terminal oligomerization domain alone is sufficient to disrupt normal wild type p53 function (25, 26). In view of the somewhat fragmentary and at times apparently contradictory information obtained thus far pertaining to the mechanistic aspects of the p53 dominant negative effect (reviewed in Refs. 18 and 19), a unifying concept that can accommodate most, if not all, of the observations made to date is badly needed. Such information would provide a better understanding of the role of p53 mutants in tumor development and would lead to the design of more precise and effective therapeutic measures in restoring wild type p53 function.

We approached this problem by first studying the biogenesis of p53 in vitro. We demonstrate here that dimerization of p53 occurs on the polysome (i.e., cotranslationally), whereas tetramerization (dimer-dimer interaction) occurs in solution (i.e., posttranslationally). Thus, coexpression of wild type and mutant p53 results in only a single heterotetrameric species of p53 (wild type dimer/mutant dimer), representing 50% of total p53. Through the use of hot spot mutants of p53 (none of which is capable of strong binding to DNA) and a variety of natural p53 target sites, we show that all heterotype interactions bind poorly to these sites compared with the wild type p53 tetramer. Such impaired binding is not likely due to mutant-imposed conformational change of wild type p53 but rather is due to the lack of complementary binding by the mutant dimeric subunit (positive dimer-dimer cooperativity). We propose this “incompetent partner” model, which reconciles a number of puzzling obser-

vations to date, as the basis of the p53 dominant negative effect. The dominant negative effect is more pronounced with sequences linked to apoptotic response than those responsible for cell growth arrest, supporting the notion that the lack of apoptotic cell death likely plays a more important role than does loss of cell growth control in cancer development.

**Experimental Procedures**

**Cloning**—The full-length constructs used were pGEMhp53wtB (wild type tetrameric human p53-hp53) and pGEM A344 (dimeric human p53-A344), both gifts from T. Halazonetis (27). hp53AN and A344AN were created by PCR with the following primers: 5’-ATATGAATTC-AACCCACGACTCTCCGACATGTCT-3’ and 3’-GGGATATCACCTCGCCCTTGCACC-3’. All p53 mutants used were also a kind gift from T. Halazonetis.

**In Vitro Transcription and Translation**—RNA was made by linearizing purified plasmid DNA with HinIII and then transcribing with Sp6 polymerase using the Megascript kit (Ambion). For translation, small aliquots of RNA were added to rabbit reticulocyte lysate (Promega). A typical reaction consisted of 17.5 μl of rabbit reticulocyte lysate, 4.5 μl of diethyl pyrocarbonate-treated H2O, 1 μl of amino acids minus methionine, 1 μl of [35S]methionine (0.5 MBq/μl), and 1 μl of RNA (typically 25 ng/μl). For situations when unlabeled p53 was used, a complete amino acid mixture was added to the translation reaction with no [35S]methionine. Translations were carried out at 37° C for 12 min.

**Immunoprecipitations**—Aliquots of translation mixture were diluted 1:5 in ice-cold phosphate-buffered saline and incubated on ice with 1 μl of the p53 monoclonal antibody DO-1 (Santa Cruz) or an equivalent amount of normal mouse IgG (Santa Cruz). 50 μl of inactivated *Staphylococcus A* (IgSorb; The Enzyme Center) was then added to the mixture. In situations where DO-1 was added to supershift the p53-DNA complex, an additional 0.25 μl of DO-1 (Santa Cruz) was included in the mixture. The reactions were incubated at 22 °C for 45 min, then cooled to 4 °C, and electrophoresed in a high ionic strength, nondenaturing polyacrylamide gel (11). The gels were dried and exposed to X-Omat Blue XB-1 film (Kodak) at 70° C in a dark room for 1 h. The gels were then boiled for 5 min prior to their electrophoresis on 10% polyacrylamide gel, 5% v/v glycerol, 0.01% bromphenol blue). The samples were cut into four times in wash buffer (400 mM NaCl, 50 mM Tris (pH 7.5), 1 mM nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and resuspended in protein sample buffer (50 mM Tris (pH 6.8), 1% SDS, 2% β-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue). The samples were then boiled for 5 min prior to their electrophoresis on 10% polyacrylamide gels containing SDS. The gels were fixed in 10% acetic acid and 15% methanol, embedded with diphénylloxazole (Sigma), dried, and exposed to X-Omat AR film (Kodak) at −70 °C. Quantification was performed on unaltered images with SigmaGel software (SPSS Science) (see Fig. 4B).

**DNA Binding Analysis**—A typical DNA binding reaction contained 2.5 μl of translation mixture, 1.2 μl of glycerol, 1 μl of salmon testes DNA (0.1 μg/μl), 0.4 μl of bovine serum albumin (50 μg/μl; Sigma), 0.25 μl of dithiothreitol (0.1 mM; Sigma), 0.25 μl of pAb421 (Oncogene Science), 2.9 μl of Tris-buffered saline, and 1 μl of [3H]-labeled DNA (1 ng/μl). In situations where DO-1 was added to supershift the p53-DNA complex, an additional 0.25 μl of DO-1 (Santa Cruz) was included in the mixture. The reactions were incubated at 22 °C for 45 min, then cooled to 4 °C, and electrophoresed in a high ionic strength, nondenaturing polyacrylamide gel (11). The gels were dried and exposed to X-Omat Blue XE-1 film (Kodak) at −70 °C. Quantification was done using a Storm 860 PhosphorImager (Molecular Dynamics) and ImageQuant software (see Fig. 7) or with SigmaGel software (SPSS Science) on unaltered images (see Fig. 4C). The sequences of CON and H1 were reported previously.
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To further confirm that p53 dimerization is a cotranslational event, we made use of another dimeric construct, A344ΔN (Fig. 2A). A344ΔN is derived from A344 and has a small deletion at the N terminus (residues 2–30), making it migrate faster than A344 upon SDS-PAGE. Like A344, A344ΔN bound the consensus DNA half-site as dimeric p53 (Fig. 2B). Furthermore, because of this deletion, A344ΔN was unable to interact with the anti-p53 antibody DO-1, which readily recognized A344 (Fig. 2C, lanes 1–6). Because DO-1 is capable of recognizing a monomer within a dimer (see below), it was used in coprecipitation studies to see whether cotranslation of the two constructs could lead to the formation of A344/A344ΔN heterodimers detectable by this antibody. The results show that under translation conditions using normal concentrations of RNA, where the two constructs formed dimers readily (as assessed by EMSA), DO-1 precipitated A344 but not A344ΔN (Fig. 2C, lanes 7 and 8). These data strongly suggest that p53 dimers form cotranslationally. To rule out the possibility that A344/A344ΔN heterodimers are unstable or undetectable, we cotranslated the two transcripts at high RNA concentrations. Under these conditions, space constraint allowed some nascent polypeptide chains from neighboring transcripts to interact (28), resulting in a small amount of A344ΔN complexing with A344 and precipitable with DO-1 (Fig. 2C, lanes 9 and 10). This shows that the lack of detection of A344/A344ΔN heterodimers under translation conditions using normal concentrations of RNA is not due to instability of the A344/A344ΔN heterodimers. It also demonstrates that the antibody DO-1 is capable of interacting with a monomer within a p53 dimer.

p53 Tetramerization Occurs Posttranslationally—We then proceeded to determine whether p53 tetramerization occurs cotranslationally or posttranslationally. The approach was similar to that above except tetrameric rather than dimeric p53 constructs (i.e. without the A344 mutation) were used. We first translated various amounts of wild type human p53 (hp53) in vitro and assessed the formation of dimeric and tetrameric p53 by direct half-site DNA binding using EMSA. Fig. 3 shows that at low transcript (and hence low protein) concentrations, the p53 made was mostly in the dimeric form. At higher transcript concentrations, the ratio of tetramer/dimer increased dramatically. This is consistent with the notion that whereas p53 dimers are formed cotranslationally, tetramers are formed posttranslationally.

To further demonstrate this point, cotranslation experiments were carried out using the wild type hp53 construct and the truncated construct hp53ΔN (Fig. 4A). When the two constructs were translated separately, DO-1 precipitated wild type p53 (hp53) but not the truncated construct hp53ΔN (Fig. 4B, lanes 1–6). When the two transcripts were mixed at approximately equimolar ratios and then translated, heterotetramers formed readily as detectable by DO-1 (Fig. 4B, lanes 7 and 8). Furthermore, the ratio of hp53 to hp53ΔN following immunoprecipitation with DO-1 was -2:1 (after normalizing to expression levels), precisely what would be expected for dimers of each species randomly oligomerizing in solution to form tetramers (see below). These results therefore again suggest that tetramerization (dimerization of dimers) is a posttranslational process.

If p53 assembly involves cotranslational dimerization followed by posttranslational tetramerization, then cotranslation of equal molar amounts of hp53 and hp53ΔN should only generate three tetrameric species, (hp53)4, (hp53)2/(hp53ΔN)2, and (hp53ΔN)4 (ideally in the molar ratio of 1:2:1). These species should be clearly discernible using the antibody DO-1 for supershift experiments in EMSA. The results of such an experiment are shown in Fig. 4C. When translated separately, hp53, but not hp53ΔN, was supershifted by DO-1 (Fig. 4C, compare lanes 4 and 6). Cotranslation of hp53 and hp53ΔN transcripts yielded a total of three species, two of which could be supershifted by DO-1 (Fig. 4C, compare lanes 7 and 8). These results...
are again consistent with the cotranslational dimerization/posttranslational tetramerization model. As mentioned above, the molar ratio of the three species should ideally be 1:2:1 (assuming proteins from the two constructs were synthesized in equimolar amounts). However, we consistently found (hp53)_4 to be somewhat under-represented (Fig. 4C, lane 8, top band). This was likely due to DO-1 partially blocking the binding of (hp53)_4 to the consensus sequence, thereby reducing the level of (hp53)_4-bound DNA (Fig. 4C, compare lanes 3 and 4). A similar observation has been previously reported for the antibody PAb246, which recognizes wild type murine p53 (11). The binding of the (hp53)_2/(hp53ΔN)_2 tetramer to DNA was apparently unaffected by DO-1, possibly because only one of the two dimers interacted with the antibody. Overall, the results from the two sets of experiments involving dimeric and tetrameric constructs, respectively, are consistent with the idea that p53 biogenesis is a two-step process: cotranslational dimerization followed by posttranslational tetramerization. This is in agreement with previous results on the folding of peptides from the tetramerization domain of p53 (29).

Dimers within Tetramers, but Not Monomers within Dimers, Are Exchangeable—Our demonstration that dimers are formed cotranslationally suggests that posttranslational exchange of monomeric subunits between dimers is probably not very efficient, if it occurs at all. On the other hand, because dimer-dimer interaction (tetramerization) is a posttranslational event, there is a good possibility that dimeric subunit exchange between p53 tetramers can occur with a certain degree of efficiency (30). This is an important consideration because it would imply that an equilibrium could exist between dimers and tetramers and that external factors or parameters could influence this equilibrium and hence p53 function.

To test this hypothesis, it was first necessary to confirm the relative lack of monomeric exchange between dimers. 35S-Labeled A344 and A344ΔN were translated separately or together in the presence of [35S]methionine. Identical fixed volumes of each 35S-labeled translation mixture were analyzed by SDS-PAGE for total hp53 protein expressed (upper panel) and by EMSA for dimeric and tetrameric hp53 through its binding to a 32P-labeled half-site (H1) (lower panel).
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To determine whether dimer/dimer exchange can take place between tetramers, a similar experiment was performed with hp53 and hp53ΔN (Fig. 5B). After a 2-h incubation period, there was a clear increase of heterotetramer formation. This shows that dimers within a tetramer of p53 can exchange with one another and that they are in equilibrium. This equilibrium strongly favors tetramers over dimers because the vast majority of p53 present following translation and maturation is tetrameric (11). Our demonstration of dimer exchange between tetramers is again congruent with the concept of p53 tetramerization being a posttranslational process involving the dimerization of dimers.

In Wild Type/Mutant p53 Heterotetramers, the Mutant p53 Dimeric Subunit Does Not Completely Abrogate Binding of the Tetramer to the Consensus Sequence—It was suggested previously that in wild type p53/mutant p53 complexes, the mutant p53 subunit(s) can induce a mutant conformation in the wild type 53 subunit(s) (20), resulting in the so-called dominant negative effect. Our present demonstration that p53 tetramers are formed by posttranslational dimerization of dimers suggests that in cells (e.g. Li-Fraumeni cells) containing a wild type and a mutant p53 allele, only a single species of heterotetramers would be generated: (wt-p53)2/(mu-p53)2. Because we have recently established that the two dimers within a p53 tetramer are conformationally independent (31), it would be of interest to assess the binding of these heterotetramers to the consensus sequence (CON). To this end, we cotranslated hp53ΔN with five of the most common p53 mutants in human cancers (1), in addition to wild type hp53 as control. Of the five mutants used, three are contact (class I) mutants (named H273, W248, and Q248) whose mutations lie in amino acids that directly associate with DNA, whereas two are conformational (class II) mutants (named S249 and H175) whose mutations destabilize the structure of the core domain of p53 (9).

Immunoprecipitation of the cotranslation reactions with the DO-1 antibody reveals that like wild type p53, all five mutants bind DNA. It thus seems unlikely that in a mutant/wild type p53 heterotetramer, the mutant dimer imposes a mutant con-
formations upon the wild type dimer. This is consistent with observations by others on heterotetramer binding to DNA (32, 33) and our previous demonstration that within a p53 tetramer, the two dimeric subunits are conformationally independent (31).

Mutant/Wild Type p53 Heterotetramers Are Biased against Sequences That Govern Apoptosis Rather Than Cell Growth Arrest—The relatively strong binding of the five heterotetramers to the CON sequence led us to wonder whether the case with CON was the exception rather than the rule. To address this issue, we chose a wide range of natural human p53 target sites (Table I). These were taken from genes involved in cell cycle arrest (p21 and 14-3-3σ), apoptosis (cyclin G, Fas, PIDD, IGF-BP3, and bax), DNA repair (gadd45), and p53 stability (hdm2), whose transcription was found to occur in a p53-dependent manner. The CON sequence was included as a reference and control. As shown in Fig. 7 (left panels), cotranslation of hp53 and hp53ΔN (the wild type control) generated three tetrameric species (hp53ΔN, (hp53)2/((hp53ΔN)2, and (hp53ΔN)2), all of which bound to each of the 10 sequences in the approximate ratio of 1:2:1 (the slight variation in migration rates between target sequences was due to size (charge) differences of these sequences). However, cotranslation of the five mutants with hp53ΔN revealed a very different scenario. First, none of the mutant homotetramers were capable of efficient binding to any of the target sites (i.e. the equivalent of the uppermost band shown in the control ((hp53)2) was either weak or not present at all in the mutant/hp53ΔN cotranslations). This was not unexpected because all of the mutants were known class I or class II mutants. Second, and importantly, all five mutant p53/hp53ΔN heterotetramers bind the 10 target sites relatively poorly compared with the wild type p53/hp53ΔN heterotramer, although the binding to CON was the least affected (Fig. 7, right panels). Reduced binding capacity of the heterotetramer was less pronounced for the mutant H273 but was clearly manifest for the other four mutants (H175, S249, Q248, and W248) (note different scales used). We reason that the reduced binding to these natural p53 target sequences is likely due to the lack of significant input in the form of complementary (and cooperative) binding from the mutant dimeric partner. A third interesting observation pertains to the extent of reduced binding of the heterotetramers to the different sequences. In particular, all of the mutant heterotetramers show drastically reduced binding to promoter sequences that govern apoptosis, namely, PIDD, IGF-BP3, and bax, compared with the other promoters, such as p21, that regulate cell cycle progression.

FIG. 6. Mutant p53 dimers oligomerize with hp53ΔN dimers, and the hp53ΔN dimer retains its ability to bind DNA. A, hp53ΔN and various class I (H273, W248, and Q248) and class II (S249 and H175) p53 mutants were cotranslated in rabbit reticulocyte lysate in the presence of [35S]methionine. They were then subjected to immunoprecipitation with a control (CTRL) antibody or the p53 monoclonal antibody DO-1. EXP represents a sample of translation reaction removed prior to immunoprecipitation to monitor protein expression. The asterisk represents a truncated p53 protein produced during in vitro translations caused by an alternate start site at codon 40. The ratios of H273, W248, Q248, S249, and H175 to hp53ΔN relative to their expression levels are 1.8:1.0, 2.0:1.0, 1.8:1.0, 2.0:1.0, 2.0:1.0, respectively. B, p53 mutants were translated separately or together with hp53ΔN in rabbit reticulocyte lysate. Translation reactions were then subjected to EMSA analysis by binding to a 32P-labeled consensus site (CON) and supershifted with the p53 monoclonal antibody DO-1 where indicated to separate different tetrameric species.

DISCUSSION

It is generally accepted that the p53 tetramer is a dimer of dimers. However, precisely how this comes about has been an enigma. It has been assumed that p53 comes off the polysome as monomers, which then dimerize in solution to form dimers; dimer-dimer interaction in turn leads to the formation of tetramers (34, 35). The present study shows that this scenario is incorrect; we demonstrate that p53 dimerization occurs cotranslationally (i.e. on the polysome), whereas tetramerization occurs posttranslationally (i.e. in solution) (Fig. 8A). In normal cells in which both p53 alleles are wild type, whether dimerization occurs cotranslationally or posttranslationally probably has no major theoretical ramifications. However, this is not the case where the cell harbors a wild type and a mutant p53 allele. Based on a posttranslational dimerization/posttranslational tetramerization mechanism (the currently accepted model), wild type p53 tetramers in such cells would comprise only ¼ of total p53. On the other hand, the alternative mechanism (cotranslational dimerization/posttranslational tetramerization) would result in ¼ of total p53 in these cells being wild type.
FIG. 7. Heterotetramer DNA binding varies depending on the mutation in one dimer and the target site. hp53ΔN was translated with hp53 or various class I (H273, Q248, and W248) or class II (S249 and H175) p53 mutants in rabbit reticulocyte lysate. Samples from each translation reaction were subjected to EMSA analysis by binding to various 32P-labeled target sites as indicated (left panels) (Table I). Radioactivity counts for each 32P-labeled target site were equalized prior to use. After film exposure, the dried gels were used for PhosphorImager analysis to quantify heterotetramer band intensity (right panels). hp53/hp53ΔN bands served as controls, and their intensity was set at 100%. Quantified mutant-hp53/hp53ΔN heterotetramer bands are expressed as a percentage of hp53-hp53ΔN band intensity. The experiments were performed three times, and the average band intensity with standard deviation is shown.

tetramers, a 4-fold difference that may well be sufficient to alter the fate of these cells upon exposure to genotoxic stress. This is an important consideration in view of the recent observation that the mere reduction in p53 levels is sufficient to promote tumorigenesis (17). Our results are compatible with the cotranslational dimerization/posttranslational tetramerization model.

In concluding that p53 dimerization occurs cotranslationally, we are suggesting that neighboring nascent p53 chains on a polysome interact with each other prior to being released into the cytosol (Fig. 8A). Such a dimerization scheme would be highly efficient because it would spare individual p53 subunits the need to search for their “partners” in a soluble pool. Precisely how monomeric p53 nascent chains interact with each other is unclear at present. Based on our current knowledge of the p53 dimerization domain, which is located at the C terminus of the protein (residues 323–356 (30, 36–38)), it would seem logical to deduce that interaction between neighboring chains occurs during the late stages of the translation process, close to the 3’ end of the p53 transcript and just prior to release of the p53 chains from the polysome. However, one cannot rule out the possibility that interaction between neighboring nascent chains first occurs at more upstream sites, which then gives way to a more stable interaction at the C-terminal oligomerization domain as soon as this becomes feasible. Considering the highly hydrophobic nature of the oligomerization domain (30, 36–38), cotranslational dimerization would offer an efficient means by which hydrophobic residues from neighboring chains could quickly interact such that protein misfolding would be greatly minimized.

In light of the model presented for the biogenesis, it is possible that the tetramerization process could represent a form of functional regulation. Assuming that p53 is imported to the nucleus as a dimer, an attractive hypothesis would be that dimeric p53 represents a latent, inactive form of p53 requiring a signal to induce tetramerization and activation. Then, following activation, degradation could be achieved by once again forming dimers to reveal a nuclear export sequence hidden in the tetramerization domain (34, 39, 40). One way of accomplishing this could be through the posttranslational modification of p53. To date, numerous posttranslational modifications have been described (reviewed in Ref. 8). In vitro, phosphorylation of Ser-315 and Ser-392 have been shown to affect p53 tetramerization (41, 42). Others have suggested potential roles for SUMOylation and ubiquitination in p53 activation and degradation by altering its oligomeric state (39, 40, 43, 44). Further experimentation is needed to properly address this issue.

Perhaps the most important conclusion from these studies pertains to the so-called p53 dominant negative effect whereby a mutant p53 protein somehow negatively affects the function of the wild type counterpart (reviewed in Refs. 18 and 19). Our results suggest that in cells possessing a wild type and a mutant p53 allele, there is only one type of heterotetramer produced, namely wild type dimer/mutant dimer. The mutant dimeric subunit within such a heterotetramer does not exert its negative effect by totally abrogating DNA binding of the complex. Based on results from the present study and our previous observation that the two dimers within a tetramer are conformationally independent (31), we favor the idea that the mutant dimer does not cause a conformational change in the wild type dimer. Rather, the lack of significant contribution (positive cooperativity) from the mutant partner leads to the overall weakened DNA binding of the heterotetramer (Fig. 8B). Because such heterotetramers bind DNA stronger than wild type dimers alone, it is likely that some cooperativity still exists and
that different target sites require varying degrees of dimer-dimer cooperativity within a p53 tetramer for DNA binding. Our model (the “incompetent partner” model) also explains why small polypeptides corresponding to the p53 oligomerization domain alone could interfere with wild type p53 function (25, 26) (because they block wild type p53 tetramerization but not dimerization) and that mutants without a functional tetramerization domain are not oncogenic (22) (because they cannot interfere with wild type p53 tetramer formation).

Although the present study clearly demonstrates that essentially all the wild type/mutant heterotetramers manifest reduced DNA binding affinity compared with the wild type protein, the extent of this reduction varies greatly and depends on both the mutant and the target sequence. Of the five mutants examined, H273 appears to exert the least dominant negative effect over the wild type protein. For example, its presence in the heterotetramer has little or no affect on the binding of the latter to the CON or p21 sequence, whereas the other heterotetramers bind to these sequences with greatly reduced efficiency. This could explain why, unlike other p53 mutants, ectopically expressed mutant H273 is still capable of CON-dependent transactivation (45–47). With the other sequences, however, H273 exerts a clear dominant negative effect on the wild type protein. But here again, this effect is not as pronounced as those manifested by the other mutants. It is interesting that H273, being the second most common p53 mutation in human cancer, is the least dominant negative by most accounts. Our results indicate that mutants W248 and Q248 are the most dominant negative mutants of the five, which could in turn explain why Li-Fraumeni patients with mutations at Arg-248 rarely display loss of heterozygosity (48); apparently Arg-248 mutation in one allele is sufficient to incapacitate wild type p53 from the normal allele.

Another striking observation pertains to the target sequences. For all the five mutants used, the p21 sequence that regulates cell growth arrest is by far the least sensitive to the dominant negative effects of these mutants. In sharp contrast, sequences that regulate apoptosis such as bax, PIDD, and IGF-BP3 all manifest very low affinity for the heterotetramers. Additionally, we found the half-lives of the p21-heterotetramer complexes to be markedly longer (by 10–30-fold) than those of bax-, PIDD-, or IGF-BP3-heterotetramers (data not shown). This bias was also demonstrable using wild type p53 (data not shown). Such differential binding affinity explains recent transactivation studies showing that p53 mutants were more dominant negative for induction of apoptosis than for growth arrest in human cancer cell lines (49). It also shows why mutants are not dominant negative in vivo when assayed for p53-dependent growth arrest (50, 51).

In summary, by studying p53 biogenesis in vitro, we demonstrate for the first time that p53 dimerization occurs cotranslationally, whereas dimer-dimer interaction (tetramerization) occurs posttranslationally. Thus in cells possessing a wild type and mutant p53 allele, only one type of heterotetramers is present: wild type dimer-mutant dimer. These heterotetramers manifest reduced binding affinity for all p53 target sequences tested, not because the mutant dimer necessarily imposes a mutant conformation on the wild type dimer, but because it has low affinity for DNA and therefore cannot significantly contribute to the overall stability of the p53-DNA complex. Reduced affinity was particularly marked for sequences that govern apoptosis rather than cell growth arrest, suggesting that defects in apoptotic cell death probably play an important role in cancer development in Li-Fraumeni patients.

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