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Complementation of two mutant p53: Implications for loss of heterozygosity in cancer

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Abstract Remarkably, a cancer cell rarely possesses two mutant p53 proteins. Instead, mutation of one allele is usually associated with loss of the second p53 allele. Why do not two mutant p53 co-exist? We hypothesize that two different p53 may complement each other, when expressed at equal levels. By titrating trans-deficient and DNA-binding-deficient p53 in cells with mutant p53 and by co-transfecting distinct mutant p53 in p53-null cells, we demonstrated activation of p53-dependent transcription. We suggest that, due to complementation of two mutant p53, cancer cells need to delete the second p53 allele rather than mutate it.

Keywords: Cancer; p53; Tumor suppressor

1. Introduction

Inactivation of the p53 tumor suppressor is the most common alteration in human cancer [1,2]. Usually, mutant p53 is associated with the loss of the second p53 allele, known as loss of heterozygosity (LOH) [3]. Wild-type (wt) p53, a transcription factor, transactivates numerous genes that cause growth arrest and apoptosis [1–7]. Also, wt p53 induces Mdm-2, which in turn targets p53 for degradation [8,9] (Fig. 1A). Since mutant p53 cannot induce Mdm-2, mutant p53 is not degraded (stable) and accumulates (Fig. 1B). Mutant p53 is stable only in the absence of wt p53 allele (Fig. 1). In the presence of wt p53, mutant p53 is unstable, because wt p53 induces Mdm-2, which in turn degrades both wt and mutant p53s [10–13], which then are equally increased (Fig. 1C). Although, when overexpressed by transfection, an ectopic mutant p53 inhibits wt p53 (dominant-negative effect), an endogenous mutant p53 cannot inhibit wt p53 [11]. In fact, mutant and wt p53 form tetramers, which retain half of p53 activity [14]. Furthermore, when in excess, wt p53 is dominant over mutant p53 [12,15–18]. Therefore, the second p53 allele must be either mutated or deleted. DNA damaging carcinogens frequently cause point mutations [19]. Furthermore, mutant p53 can exert dominant-positive effects by competing for transcriptional co-activators and by trans-activating additional genes [20–25]. Thus, it is expectable that a cancer cell may end-up with two mutant p53. However, inactivation of both p53 alleles by mutations is extremely rare. There is only one well-known cancer cell line with two mutant p53s: DU145 (prostate cancer cell line), which expresses p53-223Leu and p53-274Phe [26,27]. In other cases, one p53 is mutant and the other p53 is deleted (LOH). Why do not two p53 mutants co-exist? In order to trans-activate, wt p53 proteins form a tetramer that binds DNA [28,29]. Also, mutant p53 form active tetramers with wt p53 [14] and enhance the transcriptional activity of wt p53 [30]. When translated together with p53 lacking trans-domain (del 1–25), DNA-binding-deficient p53 can bind DNA [14]. We hypothesize that, when co-expressed, such mutant p53s regain a wt trans-activation function.

On the other hand, two mutant p53 that do not compensate each other (or, in contrast, cooperate to gain oncogenic functions) can co-exist in a cancer cell. For example, 223Leu and 274Phe cooperate to gain oncogenic functions, thus explaining why DU145 cells possess two mutant p53s [27]. This exception just confirms the rule.

Finally, conformation of mutant p53 (and wt p53) is flexible and can be influenced by interaction with other molecules, including Hsp90 [26,31], small molecules [32–34], peptides [35,36] and co-expressed p53s [37]. 273H, a DNA contact mutant p53, has wt conformation and may in theory change conformation of 173H (mutant-conformation p53). Despite these circumstantial reasons, direct evidence that two mutant p53 can actually trans-activate was missing. Here we address this question.

2. Materials and methods

2.1. Cell lines

Human prostate cancer cell lines, DU145 and PC3M; human breast cancer cell lines, SKBr3 and MCF-7 were obtained from American Type Culture Collection (Manassas, VA) and used previously [12,26,38].

2.2. Immunoblot

Proteins were harvested in TNESVF buffer and equal quantities of proteins were resolved by gel electrophoresis on either 7.5% SDS–PAGE or NuPAGE 4–12% Bis-Tris gel with MOPS running buffer (NOVEX, San Diego, CA) according to manufactures instructions. Immunoblotting was performed using mouse monoclonal antihuman p21 (Oncogene Res., Calbiochem), antihuman p53 (Ab-6 and Ab-2, Calbiochem, Cambridge, MA). Secondary antibody was anti-mouse

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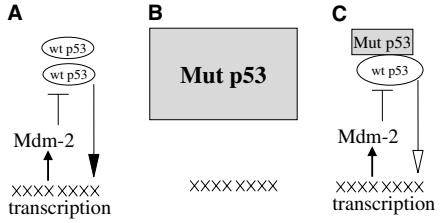

Fig. 1. Loss of the second p53 allele is necessary to lose p53 function and to stabilize mutant p53. (A) Wt p53 (two wt alleles) transcriptionally induces Mdm-2, which targets p53 for degradation. Levels of wt p53 are low (small p53). (B) One p53 allele is mutant, the second allele is lost (LOH). Mutant p53 is not degraded because it cannot transactivate Mdm-2. Therefore, mutant p53 is accumulated at high levels (big p53). (C) One p53 allele is mutant, the second allele is wt. Wt p53 induces Mdm-2, which degrades both wt p53 and mutant p53. Both mutant p53 and wt p53 are expressed at similar (low) levels.

or anti-rabbit Ig horseradish peroxidase-linked (Amersham, Piscataway, NJ). The membrane was developed using ECL™ Western blotting detection reagents (Amersham).

2.3. Transient transfection

WWP-Luc, a p21 promoter-luciferase construct, and PG13-Luc, containing 13 generic p53 response elements were obtained from Dr. El-Deiry [39]. The control luciferase plasmid, pGL2-Control, driven by SV40 promoter and enhancer sequences, was purchased from Promega (Madison, WI). pCMV–galactosidase was purchased from Clontech (Palo Alto, CA). Wt p53, 175His, 273His mutant p53 were obtained from Dr. Vogelstein. The p53 expression plasmid consists of wt or mutant p53 cDNA cloned into the *Bam*HI site of pREP4 (Invitrogen).

50×10^3 and 200×10^3 cells/well were plated in 24- and 6-well plates, respectively (Costar, Acton, MA). The next day, cells were transfected with plasmids in the presence of Lipofectamine (Gibco-BRL, Gaithersburg, MD) or with TransFast Transfection Reagent (Promega) according to manufacture recommendations. 6 h later, the medium was changed for additional 24 h, unless otherwise indicated. Luciferase activity was measured as described previously [12].

3. Results

3.1. Trans-deficient p53 induces transcription in DU145 and SKBr3 cells

To address the question whether two mutant p53 can complement each other (compensate for the loss of functions), we transfected DU145 cells using transactivation-deficient mutant p53s: namely, p53 with a double-point mutation 22/23 (p53-22/23) and p53 lacking 25 amino acids on the N-end (p53-N25-del). The NH2-end of p53 is a transactivation domain [40]. These mutant p53 cannot bind a transcription co-activator p300/CBP. Both mutant p53 still bind DNA. DU145 cells contain two endogenous mutant p53, which in contrast cannot bind to DNA. DU145 cells were chosen to maximize the chances that at least one endogenous mutant p53 will interact with p53-22/23 and p53-N25-del. We expected that the p53 transcription factor may bind DNA and transactivate via transfected and endogenous p53.

We found that transfection with 8–40 ng plasmid DNA/well of either p53-N25-del or p53-22/23 induced PG13-Luc in DU145 cells (Fig. 2). As a negative control, these mutants did not transactivate CMV-Luc (Fig. 2B). By increasing an amount of transfected p53, we observed a decrease in transactivation of PG13-Luc. At 1000 ng/well, both p53-22/23 and p53-N25-del suppressed PG13-Luc, returning its expression

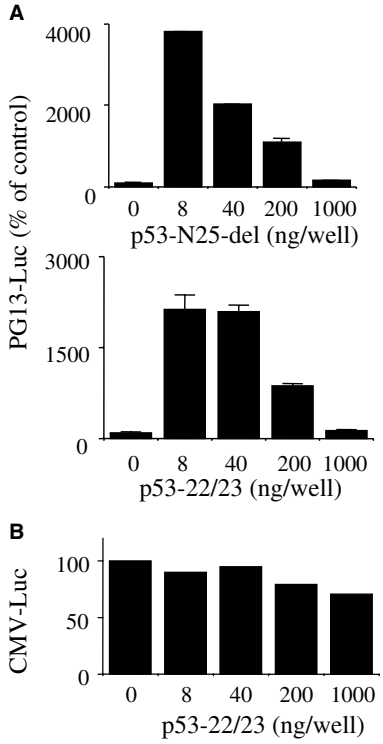

Fig. 2. Trans-activation domain deficient mutant p53-N25-del and p53-22/23 induce PG13-Luc in DU145 cells. (A) DU145 cells were co-transfected with 1 μg PG13-Luc and indicated amount (ng) of either p53-N25-del or p53-22/23-expressing plasmids. (B) DU145 cells were co-transfected with CMV-Luc and p53-22/23 mutant p53-expressing plasmid. Total amount of DNA was normalized by 2 μg with empty vector. After 16 h, cells were lysed and luciferase activity was measured.

to the basal level (Fig. 2A). Similar results were obtained in SKBr3 breast cancer cell line having 175H mutant, which is deficient in DNA binding (data not shown).

3.2. Co-transfection of N-deficient and 273H mutants transactivates PG13-Luc in PC3M

Next, we co-transfected two mutant p53 plus PG13-Luc in PC3M cells, which have no endogenous p53 (p53 null). In control, PG13-Luc was expressed at very low levels, reflecting lack of endogenous p53 in PC3M cells (Fig. 3). As a positive control, transfection with wt p53 dramatically induced PG13-Luc. 273H, a contact mutant, is one of the most common in human cancer. As expected, this mutant p53 did not transactivate PG13-Luc (Fig. 3). In PC3M, p53-N25-del and p53-22/23 display lower activities than wt p53. It was difficult to co-transfect mutant p53s at equivalent levels. Yet, when p53-N25del and 273H were expressed at similar levels, then 273H potentiated p53-N25-del (Fig. 3: N25 +273). At the same conditions, 273H also potentiated p53-22/23 (Fig. 3: 22/23 +273).

3.3. Dose-dependent reversal of co-activation in PC3M

Next, we wished to confirm that an excess of p53-N25-del can reverse co-activation of PG13-Luc (Fig. 4). We started with the condition of equal expression, as found in Fig. 3. When co-expressed at equal levels, p53-N25-del and 273H stimulated PG13-Luc (Fig. 4A and B). Then, while keeping constant expression of 273H, we increased expression of

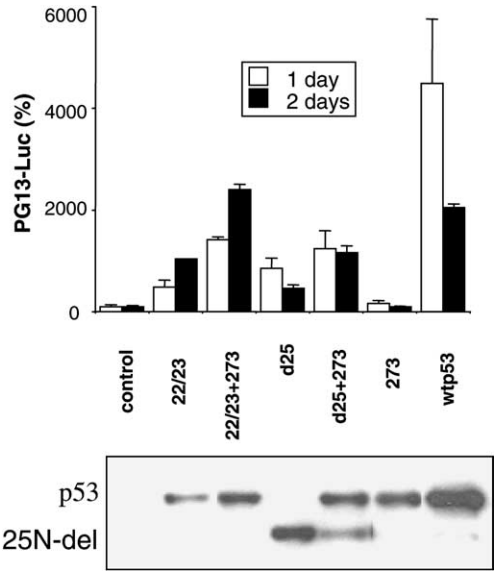

Fig. 3. Mutant 273H (273) potentiates the trans-activity of p53-22/23 and p53-N25-del. PC3M cells were transfected with 1 μg/well PG13-Luc and co-transfected with plasmids expressing indicated p53s: p53-22/23, p53-N25-del, H273 either alone (1 μg/well) or in combinations (0.5 μg/well plus 05 μg/well). Total amount of DNA is 2 μg/well. Upper panel: luciferase activity (PG13-Luc) was measured at day 1 and 2. Lower panel: p53 protein was measured by immunoblot at day 1. Wt p53, p53-22/23 and 273H are seen as full length p53. p53-N25-del, with lower molecular mass, is seen below.

p53-N25-del. By increasing p53-N25-del expression, we observed the abrogation of activation of PG13-Luc. It is noteworthy that, when transcription was activated (at 20 ng p53-N25-del plasmid DNA/well), levels of 273H were decreased, compared with either no p53-N25-del or high levels

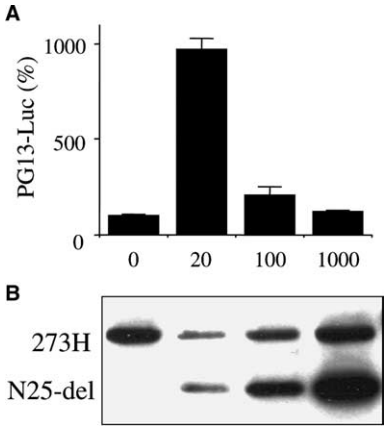

Fig. 4. Co-expression of mutant 273H (273) and p53-N25-del. PC3M cells were transfected with 1 μg/well PG13-Luc and co-transfected with 40 ng/well 273H and increasing amounts of N-del 25 (0–1000 ng/well). (A) Luciferase activity (PG13-Luc) was measured at day 1. (B) p53 protein was measured by immunoblot at day 1.

of p53-N25-del (Fig. 4B). This is consistent with degradation of mutant 273H, in the presence of the p53 function.

3.4. Co-activation by two DNA binding mutants

Finally, we wished to confirm that two natural mutant p53s, when co-expressed together, can transactivate PG13-Luc. We compared effects of wt p53 and 273H in cells lacking p53 (PC3M), cells expressing 175H p53 (SKBr3) and cells expressing wt p53 (MCF-7). Both wt p53 and 273H were expressed in PC3M in a dose-dependent manner (Fig. 5, upper panel). Wt p53 induced PG13-Luc transcription, whereas 273H did not (Fig. 5, PC3M). Also, wt p53 induced PG13-Luc transcription in SKBr3 cells. Noteworthy, at 20 ng/well, wt p53 induced a

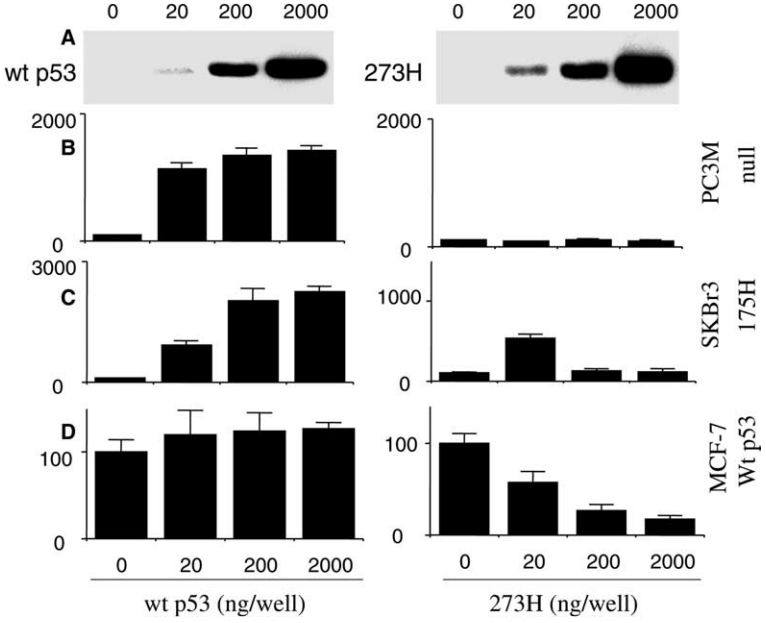

Fig. 5. Comparison of transactivation, complementation and dominant-negative effects caused by 273H. (A, B) PC3M cells were co-transfected with 1 μg/well PG13-Luc and either wt p53 (left column) or 273H (right column): 0–2000 ng/well. (A) p53 and (B) luciferase were measured after 24 h. SKBr3 (C) and MCF-7 (D) cells were co-transfected with 1 μg/well PG13-Luc and either wt p53 (left column) or 273H (right column): 0–2000 ng/well. Luciferase activity was measured after 24 h. The luciferase activity is shown as percent of control (p53 = 0) ± *m*.

near-maximal transcription in PC3M (null) cells but only a half-maximal transcription in SKBr3 (endogenous 175H) cells, due to competition between wt p53 and endogenous mutant p53 (dominant-negative effect). This actually indicates that transfection with 20 ng p53-expressing plasmid/well yields physiological levels of p53 expression. In agreement, 20 ng 273H induced PG13-Luc in SKBr3 cells, which have endogenous 175H. At higher levels, 273H did not induce PG13-Luc. In agreement, high levels of 273H exerted dominant-negative effect against wt p53 (Fig. 5, MCF-7).

4. Discussion

The tumor suppressor p53 is mutated in 50% of cancers. When one p53 is mutated, the second allele is usually deleted (LOH). Why the second allele is not mutated as well. To explain why two different mutant p53 do not usually co-exist in a cancer cell, we suggest that, when co-expressed in the same cell, two mutant p53 can regain trans-activation functions. This suggestion is not entirely surprising. Numerous transcription factors (AP-1, HIF-1, etc.) are heteromers with subunits that are not necessarily active as homomers. In this light, two different mutant p53s are 'distinct subunits' that are inactive as homotetramers but can form heterotetramers.

Here, we showed that the co-expression of DNA-binding-deficient mutants (273H or 175H) with p53 mutants that can bind DNA (p53-22/23 or p53-N25-del) results in trans-activity. We conclude that the p53 transcription factor binds DNA via p53-22/23 (or p53-N25-del) and trans-activate via 273H (or 175H). Furthermore, when co-expressed at equal levels, 273H and 175H caused transactivation of PG13-Luc. If one of the mutants is in access, it exerts dominant-negative effect. In agreement, high levels of 273H inhibited PG13-Luc in SKBr3 (175H) and in MCF-7 (wt p53) cells. The stoichiometry of the mixed complexes, under transactivating and non-transactivating conditions, can be planned for further investigations.

Obviously, two mutant p53 with identical mutations will not complement each other. The chances that two alleles are mutated at the same nucleotide, however, are low. Perhaps such identical mutations rarely occur but are perceived as one mutation, not two independent mutations. Also, 'similar' mutants are unlikely complementary. For example, in DU145 cells, two endogenous p53 mutants do not acquire wt functions but instead gain new functions [27]. Interestingly, in DU145 cells, mutant p53 are especially prone to destabilization by geldanamycin, which partially rescue wt functions [26], implying that two mutant p53 can acquire trans-functions.

If two mutant p53 do not co-exist to avoid wt function, then why 50% cancer cells have wt p53, thus retaining wt function. The simplest answer is that some cancer cells prefer wt function to start with. Such cancer cell lines are apoptosis-reluctant (due to inactivation of caspases in MCF-7 cells, for instance, and Apaf-1 in melanoma cells [41], for instance). Whereas cancer cells with mutant p53 cannot tolerate exogenous wt p53, cells with wt p53 are relatively resistant to apoptosis [18]. Cells with wt p53 may actually benefit from retaining wt p53 [42]. Yet, if a cell needs to inactivate p53, it acquires either null or LOH phenotype. In other words, if there is a selective pressure against the wt p53 function, a cell cannot end up with two mutant p53 that compensate each other.

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