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The Effects of a Mutant p53 Protein on the Proliferation and Differentiation of PC12 Rat Phaeochromocytoma Cells

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Abstract PC12 rat phaeochromocytoma cells show neuronal differentiation upon NGF treatment. NGF induces prolonged activation of the Ras/Raf/MEK/ERK pathway in which the 42/44 kDa mitogen-activated protein kinases (MAPKs), ERK 1 and 2 are thought to be the key mediators of the differentiation signals. Activation of ERKs leads to the increased transcription of early response genes resulting in cell cycle arrest. Upon NGF treatment the p53 protein, the most commonly mutated tumor suppressor in human cancers, translocates to the nucleus and may play a role in the mediation of NGF-induced cell cycle arrest and neuronal differentiation. Here we demonstrate that in PC12 cells expressing both wild-type and V143A mutant p53 proteins (p143p53PC12 cells), p53-mediated biological responses are critically influenced. p143p53PC12 cells are not able to cease their proliferation and begin their neuronal differentiation program upon NGF treatment. The presence of mutant p53 also reduces the DNA-binding activity of endogenous p53 and disturbs the regulatory machinery of p53 including both the phosphorylation of ERK 1/2, p38 and SAPK/JNK MAP kinases and itself. J. Cell. Biochem. 99: 1431–1441, 2006.

Key words: NGF; V143A p53 mutation; ERK pathway; stress signaling

p53, a widely expressed 53 kDa nuclear phosphoprotein, is one of the main regulators of the cell cycle in mammalian cells. Acting as a tumor suppressor protein, it blocks cell proliferation by inhibiting the G_1/S phase transition in response to a variety of signals. As a transcription factor, it regulates gene expression responsible for cell cycle arrest, repair of damaged DNA and induction of apoptosis [Levine, 1997]. In proliferating cells p53 is mainly sequestered in the cytoplasm by the phosphorylated ubiquitin ligase MDM2, inhibiting tetramerization and nuclear translocation of p53 needed to its proper cell cycle controlling function. Upon Akt/protein kinase B (PKB)-mediated phosphorylation of MDM2 p53 is ubiquitinated and degraded by proteasomes [Haupt et al., 1997]. During its activation, p53 is released by dephosphorylated MDM2, oligomerization takes place and p53 translocates to the nucleus. Besides MDM2, the lipid phosphatase PTEN is also reported as an important regulatory protein of p53. In contrast to MDM2, PTEN saves p53 from MDM2-mediated degradation by inhibiting the Akt/PKB MDM2 pathway: as a lipid phosphatase, it eliminates the second messenger phosphatidyl-inositol-trisphosphate (PIP_3) thereby preventing the activation of Akt/PKB protein kinase [Mayo and Donner, 2002]. The activity of p53 can also be regulated by post-translational modifications as acetylation/deacetylation and/or phosphorylation at its various serine and threonine residues by several stress or DNA damage-induced kinases such as ATM, ATR, and Chk1/2.

Abbreviations used: DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; NGF, nerve growth factor; dbcAMP, dibutyryl-cyclic AMP; PKB, protein kinase B; PIP_3, phosphatidyl-inositol-trisphosphate; LOH, loss of heterozygosity; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase.

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Different phosphorylation patterns seem to be critical in the molecular selection of the sets of genes to be up-regulated by p53 [Brooks and Gu, 2003].

The central role of p53 in the development of human cancers is underlined by the finding that almost 50% of diagnosed human tumors carry mutant forms of this tumor suppressor [Hollstein et al., 1991]. Inactivation of the p53 gene is essentially due to small mutations (missense and nonsense mutations or insertions/deletions of several nucleotides), which lead to either expression of a mutant protein (90% of cases) or absence of the protein (10% of cases). Mutations affecting the DNA-binding domain of p53 are among the most common ones identified in human cancers. Interestingly, some mutations affecting the DNA binding domain of p53, including the Val-143-Ala, Arg-175-His, Arg-248-Trp, Arg-249-Ser, and Arg-273-His mutations, can activate transcription from certain p53-regulated promoter regions at 32°C or below, but usually fail to induce the appropriate biological responses [Friedlander et al., 1996]. In many cases, p53 mutations are associated with loss of the wild-type (wt) allele of the p53 gene, leading to the loss of heterozygosity (LOH) of the second allele [Demidenko et al., 2005].

PC12 rat phaeochromocytoma cells [Greene and Tischler, 1976], a widely used cellular model system to study neuronal differentiation, proliferation or apoptosis, express wt p53 alleles [Eizenberg et al., 1996], and show neuronal differentiation upon treatment with nerve growth factor (NGF). In wtPC12 cells the 42/44 kDa mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERK 1 and 2) are thought to be key mediators of differentiation signals. NGF induces prolonged ERK activation in PC12 cells leading to transcriptional activation of the so called early response genes [Greene and Kaplan, 1995; Marshall, 1995]. Activation of ERK 1 and 2 is induced by the upstream kinases MEKs that phosphorylate threonine and tyrosine residues on ERKs. Phosphorylation of ERK 1 and 2 on threonine 202 and tyrosine 204 can be detected after minutes of NGF or EGF treatment. Previous studies also showed that p53 translocates to the nucleus 16 h after NGF treatment. It has been reported that in wtPC12 cells, p53 plays a critical role in the mediation of NGF-induced neurite outgrowth, an easily detectable response of naive PC12 cells to NGF [Eizenberg et al., 1996; Hughes et al., 2000].

The aim of our current study was to define the effects of the V143A mutant p53 protein on biological responses of PC12 cells upon a variety of treatments leading to wtp53 activation in wt cells. Here we present data that in stably transfected PC12 cells expressing both wt and V143A mutant p53 proteins (p143p53PC12 cells), p53-mediated biological responses are critically influenced. p143p53PC12 cells are not able to cease their proliferation and start their neuronal differentiation program upon NGF treatment. The presence of mutant p53 reduces the DNA-binding activity of endogenous p53 in a dominant negative manner and disturbs the regulatory machinery of p53 including the phosphorylation of both MDM2, MAPK ERK 1/2 and itself. Our data indicate that co-expression of mutant and wt p53 could be sufficient to up-regulate proliferation and to abolish the ability of cells to respond to a variety of cell cycle arresting signals.

MATERIALS AND METHODS

Materials

All biochemicals and culture media were purchased from Sigma–Aldrich Hungary (Budapest, Hungary), unless otherwise stated. NGF was purchased from Invitrogen (Carlsbad, CA).

Cell Cultures

PC12 cells were cultured in DMEM with 4.5 g/L glucose supplemented with 10% horse serum and 5% fetal bovine serum (FBS). Animal sera were purchased from GIBCO (Paisley, Scotland, UK).

Stable Transfection of wtPC12 Cells With V143A Mutant p53 cDNA

wtPC12 cells were transfected with an expression plasmid carrying a Val-143-Ala mutant p53 cDNA under the control of a cytomegalovirus promoter and a geneticin (G418) resistance gene for selection. Transfection was carried out with LipofectAMINE (GIBCO) according to the instructions of the manufacturer. After 24 h of transfection stable transfectants were selected by adding G418 (400 μg/ml) to the culture medium. Individual G418-resistant clones were isolated. Expression of the exogenous mutant p53 was confirmed by Northern blot analysis using a radioactively

labeled fragment of the mutant p53 cDNA as a probe (Fig. 1, Panel A). For our further experiments the clone showing the strongest expression of the mutant p53 gene (clone #9) was selected and cultured in medium containing G418 (200 μg/ml).

Quantification of Differentiation and Proliferation of PC12 Cells

10^5 naive PC12 cells were seeded on six-well plates and cultured for 24 h before treatment. Treatments were carried out in triplicates. Cells having two times longer neurite-like projections than the diameter of the cell body were recorded as differentiated cells. Upon the course of NGF treatment culture media were exchanged every third day. Counting was carried out on day 1, 6 and 9; at least 1,000 cells were analyzed in each well of the six-well plates. On day 9 wells were washed with fresh culture medium to eliminate dead cells, then the remaining live cells were trypsinized, collected and the total cell numbers for each well of the six-well plate were determined by haemocytometric measurements.

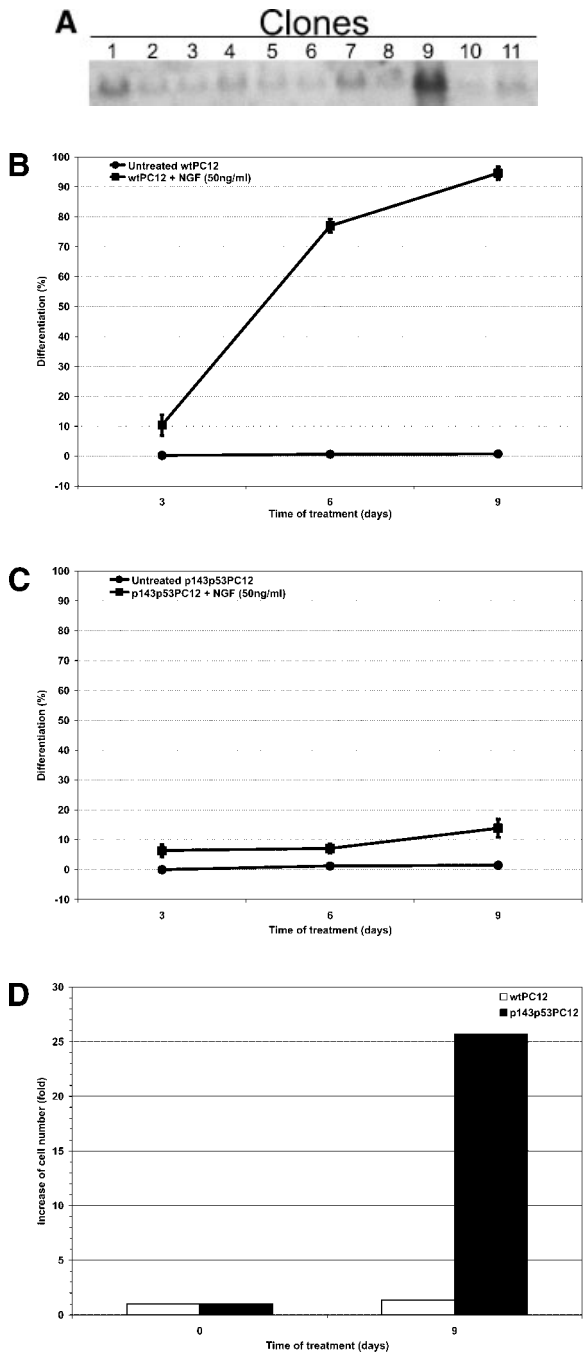

Fig. 1. Differentiation and proliferation of PC12 cells expressing wt and V143A mutant p53 protein upon NGF treatment. **Panel A** shows the result of the Northern blot confirmation of the expression of V143A mutant in different isolated clones. Samples contained 10 μg of total RNA. Ethidium bromide staining of the gels reveaed comparable amounts of 28S and 18S rRNAs in each lane. For our further experiments clone #9 was selected and used. (**B**–**C**) Cells were kept untreated (●) or treated with 50 ng/ml NGF (■) at 37°C for the indicated time periods and the ratio of differentiated cells was determined as described in Materials and Methods using both wtPC12 cells (B) and p143p53PC12 cells (C). **D**: The number of differentiated cells were counted as described in Materials and Methods on day 3, 6, and 9. After 9 days of NGF treatment the total number of cells was determined. (□) Indicates wild-type PC12 cells, (■) indicates p143p53PC12 cells.

Western Blotting

Immunoblot analysis using antibodies against proteins indicated in the figures was performed according to the manufacturers's recommendations. Cells were collected by scraping and centrifuged at 600*g* for 3 min, lysed in ice-cold lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM Na-orthovanadate, 5 μM $ZnCl_2$, 100 mM NaF, and 1% Triton X-100. The lysis buffer was supplemented with 10 μl/ml phosphatase inhibitor cocktail I and 1 tablet/10 ml protease inhibitors (Complete, Mini EDTA-free tablets, Roche Hungary, Budapest, Hungary). Following lysis for 10 min on ice, samples were centrifuged at 13,500 rpm for 10 min at 4°C. Protein concentration was determined using the Bio-Rad Protein RC_{DC} assay system (Bio-Rad Budapest, Hungary). At least thirty μg proteins for each sample were resolved by SDS polyacrylamide gel electrophoresis in 10% gels. The proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech AB., Uppsala, Sweden), treated with the appropriate antibodies and immune complexes were visualized using an Enhanced Chemiluminescence

Detection kit (Amersham Pharmacia Biotech AB.) following the manufacturer's instructions. The following antibodies were used: p53 (pAb), phospho-p53 (Ser6, pAb), phospho-p53 (Ser15, pAb), phospho-p53 (Ser20, pAb), phospho-p53 (Ser46, pAb), phospho-p53 (Ser392, pAb), phospho-p38 (Thr180/Ty182 pAb), p38 (pAb), phospho-SAPK/JNK 1/2/3 (Thr183/Tyr185 pAb), SAPK/JNK 1/2/3 (pAb), phospho-ERK 1/2 (Thr202/Tyr204 pAb) antibodies from Cell Signaling (Beverly, MA), ERK2 (pAb) SantaCruz Biotechnology (Santa Cruz, CA). Actin (mAb, AB-1) antibody was purchased from Oncogene (Merck Ltd., Budapest, Hungary).

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared as described previously [Xu and Cooper, 1995]. All subsequent steps were performed at 4°C. Cell pellets were washed twice in ice-cold PBS and resuspended in 10 volumes of 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), protease inhibitors (Complete, Mini EDTA-free tablets, Roche Hungary) and phosphatase inhibitor cocktail I and placed on ice for 10 min. Nuclei were collected by centrifugation and resuspended in 2 volumes of 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors and phosphatase inhibitor cocktail I and placed on ice for 20 min. After centrifugation, protein concentrations of the supernatants were determined by the Bio-Rad RC_{DC} Protein Assay kit. 5′-end labeling of the p53 oligonucleotide was performed using [γ-^{32}P] ATP (Institute of Isotopes Co., Ltd., Budapest, Hungary) and T4 polynucleotide kinase (Amersham Pharmacia Biotech AB.) according to the manufacturer's protocol. Double-stranded p53 oligonucleotide containing the consensus binding site for p53 (5′-TACAGAACATGTCTAAGCATGCTGG GGACT-3′) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz), double-stranded oligonucleotide containing the consensus binding site for c-Myc (5′-TGTGCG GCCACGTGTCGCGAGGCCCGG-3′) was purchased from Amitof (Boston, MA). Ten micrograms of nuclear proteins were mixed with 100 ng nonspecific single-stranded oligonucleotide in 4 μl buffer containing 10 mM HEPES (pH 7.5), 10% glycerol, 1 mM EDTA, 100 mM NaCl, 2 μg poly(dI-dC) for c-Myc or 3 μg poly(dI-dC) for p53 binding reactions and the final reaction volume was adjusted to 18 μl with distilled water. After a 15-min pre-incubation at room temperature 10^5 cpm of ^{32}P-labeled oligonucleotide was added and the incubation at room temperature was continued for another 30 min. DNA-protein complexes were electrophoresed in 5% non-denaturing polyacrylamide gels using a TRIS-Base, borate, EDTA buffer (pH 8.3) for 2.5 h at 200 V. The gel was dried and analyzed by a Cyclone PhosphorImager (Packard Instrument Co. Inc., Meriden, CT).

RESULTS

Expression of V143A Mutant p53 Blocks NGF-Induced Neurite Outgrowth of PC12 Cells

It has been known for a long while that, when NGF-treated, naive wtPC12 cells cease their proliferation and begin to differentiate toward a neuron-like phenotype. During this process p53 is responsible for stopping cell division allowing cells to complete their differentiation program [Eizenberg et al., 1996; Hughes et al., 2000]. To determine whether differentiation and inhibition of proliferation of PC12 cells upon NGF treatment is affected by the mutant form of p53 we used wtPC12 cells stably transfected with a CMV promoter-driven V143A mutant p53 cDNA (a clone designated p143p53PC12), and determined the ratio of differentiated cells upon NGF treatment. As Figure 1 indicates wtPC12 cells differentiated as expected, while those expressing the mutant p53 failed to do so. After 9 days of treatment, more than 90% of the wtPC12 cells showed the differentiated phenotype while among untreated cells the ratio of differentiated cells remained less than 5% (Fig. 1B). In contrast, p143p53PC12 cells treated with NGF at a same concentration hardly displayed any biological response to NGF treatment: after 9 days of treatment less than 20% differentiated cells could be detected (Fig. 1C). It should be noted that stably transfectant cells showed a slightly different morphology without NGF treatment than their normal counterparts: they were more polygonal with small but virtually not completed projections. Thus it seems that in p143p53PC12 cells the mutant p53 perturbs NGF-induced neuronal differentiation. Moreover, comparing the rate of proliferation capacity of cells we found that after 9 days of NGF treatment the number of

p143p53PC12 increased 25-fold of the starting number of cells, while wtPC12 cells ceased their proliferation (Fig. 1D).

Expression of V143A Mutant p53 Interferes With Enhancer-Binding of the Endogenous wtp53 Protein

Since expression of the V143A mutant p53 in PC12 cells nearly completely blocked NGF-induced differentiation and the p53 protein exerts most of its effects as a transcriptional factor, next we analyzed the p53 DNA-binding activity in the wt and transfected cells under different conditions. As Figure 2 indicates, even untreated proliferating wtPC12 cells have an easily detectable basal DNA-binding activity of p53 that can be further induced by various treatments. A short exposure of cells to dibutyryl-cyclic AMP (dbcAMP), a diffusible cAMP analogue, in a concentration inducing neurite outgrowth was sufficient to elevate p53 DNA-binding activity. Similar results were obtained with etoposide and anisomycin treatments or UV irradiation. Interestingly, etoposide, a strong topoisomerase I inhibitor causing apoptotic cell death in PC12 cells, increased p53 DNA-binding activity even after 120 min indicating that DNA damage caused by this agent stimulates p53 very rapidly in cells residing in S phase during the treatment. Treatments with anisomycin, an antibiotic agent stimulating stress signaling pathways (e.g., p38, JNK pathways) transcription in low concentration (<100 ng/ml), but apoptosis by the complete block of protein synthesis in higher concentrations (>500 ng/ml) in wtPC12 cells, led to the same results. One hundred twenty minutes of UV exposure slightly induced the DNA-binding activity of endogenous p53. On the other hand, neither a short treatment of wtPC12 cells with NGF, that is sufficient to stimulate differentiation-mediating signaling pathways, nor serum starvation, another apoptotic stimulus of wtPC12

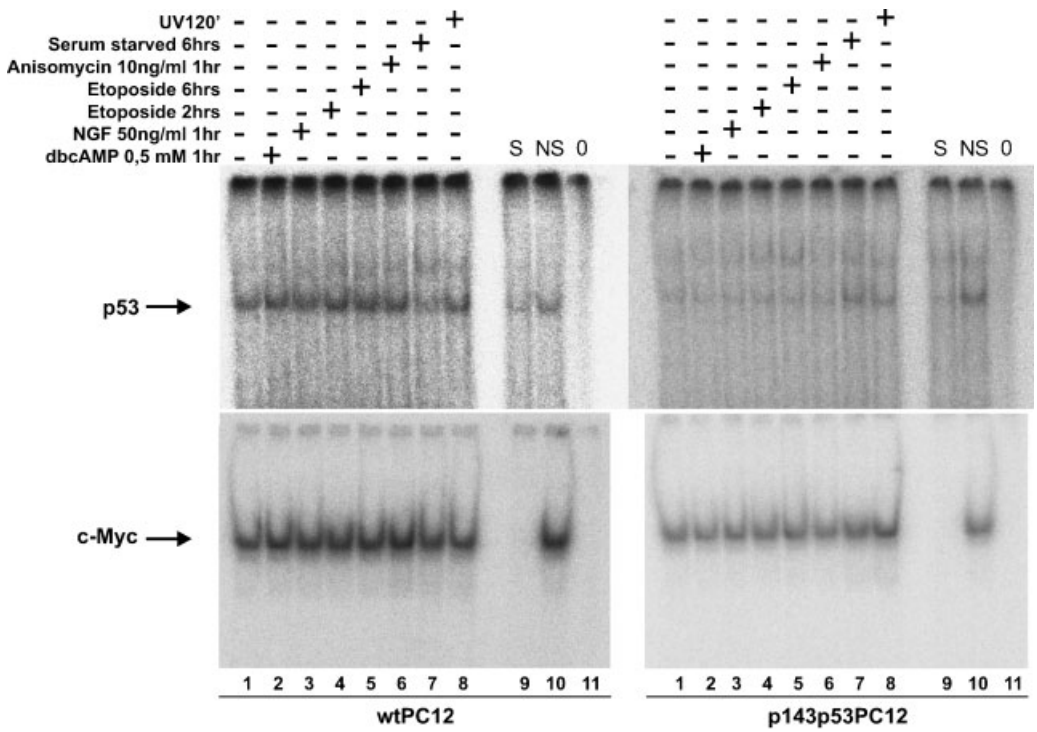

Fig. 2. DNA-binding activity of p53 and c-Myc in wtPC12 and p143p53PC12 cells. Cells were treated as indicated, nuclear proteins were isolated and subjected to electro-mobility shift assays as described in Materials and Methods. All treatments except serum starvation were carried out in DMEM culture media supplemented with 10% horse serum and 5% FBS. Treatments, except UV irradiation, were performed at 37°C. For controls untreated cells (samples 1) were used. DNA-binding reactions were performed using ^{32}P-labeled oligonucleotides carrying p53 (**upper panels**) or c-Myc consensus sequences (**lower panels**) as described in Materials and Methods. To prove the specificity of DNA-binding, unlabeled competitor oligonucleotides containing specific (labeled as ''S,'' samples 9), p53 oligonucleotide for **upper panels**; c-Myc binding oligonucleotide for **lower panels** or non-specific (labeled as ''NS,'' samples 10) AP1 oligonucleotide consensus sequences were used in excess amount. Samples 11 served as protein free controls.

cells, could elevate p53 DNA-binding capacity significantly.

In contrast to wtPC12, in proliferating p143p53PC12 cells reduction of the basal p53 DNA-binding activity to a hardly detectable level was observed indicating that the expression of the V143A mutant p53 protein interferes with the endogenous p53 activity in a dominant negative fashion. Moreover, DNA-binding activity of p53 after treatment of p143p53PC12 cells with dbcAMP, etoposide or anisomycin was not increased. One hundred twenty minutes of UV irradiation induced p53 DNA-binding in p143p53PC12 cells as well. (It should be noted that UV irradiation of cells was performed in a laminar air flow cabinet in which stable temperature could not be maintained and the temperature sensitive V143A mutant protein could have regained its DNA-binding activity due to a temperature drop.) However, slightly elevated DNA-binding activity was also detected in serum-starved p143p53PC12 cells, albeit they were kept in a cell culture incubator at 37°C during their treatment suggesting that serum withdrawal prevented the dominant inhibitory activity of the exogenous p53 proteins. Serum starvation causes G_1/S phase arrest, at least in part, by p53-dependent mechanisms, so we can assume that under these conditions endogenous p53 expression is also induced possibly shifting the wtp53/V143A mutant p53 ratio leading to higher levels of tetramers containing only wt form of the protein. To exclude a possible general reduction of transcriptional activity in p143p53PC12 cells, we analyzed c-Myc DNA-binding activity in the same nuclear protein samples. We found it comparable in both cell types and only slightly affected by the treatments used in this experiment (Fig. 2, lower panels). We can thus conclude that the expression of the V143A mutant p53 protein selectively blocked the p53-enhancer binding activity in PC12 cells.

Expression of Mutant p53 Alters the Phosphorylation State of p53 Upon Various Treatments

Post-translational modifications seem to be important events in the regulation of p53. To date several amino acid residues have been described as possible sites for phosphorylation, acetylation or ubiquitination, among which residues for phosphorylation events have been studied most extensively [Brooks and Gu, 2003]. Since we found that in PC12 cells expression of the V143A mutant p53 blocked NGF-induced neuronal differentiation, it seemed to be an obvious question if the presence of the mutant p53 protein had any effect on the phosphorylation of the most important serine residues of p53. To address this question we studied the phosphorylation state of p53 proteins both in wtPC12 and p143p53PC12 cells using phospho-specific antibodies in Western-blot assays (Fig. 3A,B). In wtPC12 cells p53 proteins could be easily detected while in p143p53PC12 cells the p53 level was somewhat reduced. In wtPC12 cells we detected slight induction of p53 expression upon NGF, dbcAMP treatments and UV irradiation (Fig. 3A, samples 2, 3, 6), while exposure of wtPC12 to etoposide induced a significant increase in p53 level (Fig. 3A, samples 7, 8). On the other hand, serum starvation led to a slight decrease in the intracellular p53 level (Fig. 3A, samples 5). In contrast to the wtPC12 cells, in p143p53PC12 cells both NGF and dbcAMP failed to increase the amount of the p53 (Fig. 4A, samples 2, 3,), while in transfectant cells exposed to UV irradiation or etoposide treatment significant induction was still observed (Fig. 3, samples 6–8). Analyzing the status of the main phosphorylation sites of p53

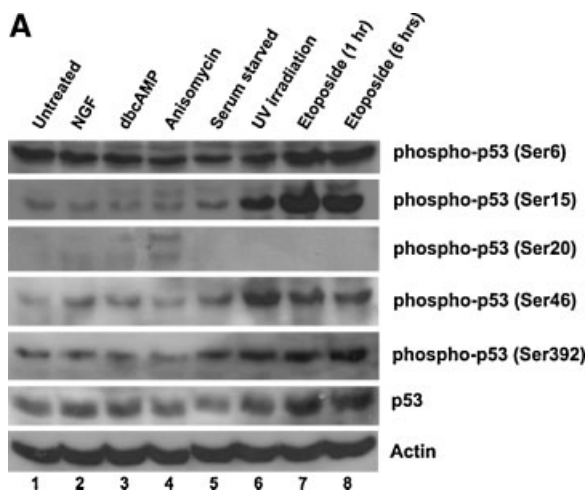

Fig. 3. Phosphorylation of p53 in wtPC12 (**panel A**) and p143p53PC12 cells (**panel B**) after various treatments. Cells were treated with NGF (50 ng/ml, for 1 h), dbcAMP (0.5 mM, for 1 h), anisomycin (10 ng/ml for 1 h), etoposide (50 μM for 1 or 6 h), or exposed to UV irradiation or serum starvation for 2 or 6 h, respectively. All treatments, except serum starvation, were carried out in DMEM culture media supplemented with 10% horse serum and 5% FBS. Treatments, except UV irradiation, were performed at 37°C. Whole cell lysates were subjected to immunoblot analysis with antibodies against p53, actin (as loading control) and phosphorylated forms of p53.

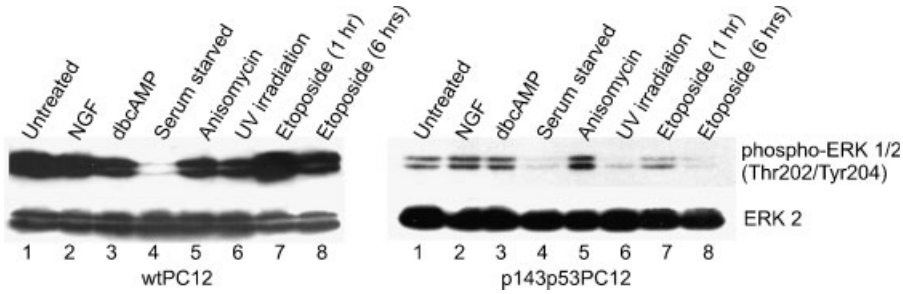

Fig. 4. Phosphorylation of ERK-1 and -2 in wtPC12 and p143p53PC12 cells after various treatments. Cells were treated with NGF (50 ng/ml, for 1 h), dbcAMP (0.5 mM, for 1 h), anisomycin (10 ng/ml for 1 h), etoposide (50 μM for 1 or 6 h), or exposed to UV irradiation for 2 h or serum starvation for 6 h. All treatments, except serum starvation, were carried out in DMEM culture media supplemented with 10% horse serum and 5% FBS. Treatments, except UV irradiation, were performed at 37°C. Whole cell lysates were subjected to immunoblot analysis of ERK proteins.

we found that the phosphorylation pattern of p53 was generally the same in the untreated wt and transfectant cells (Fig. 3, lanes 1). Similar results were observed upon NGF treatment (Fig. 3, lanes 2) suggesting that the lack of NGF-induced neurite-outgrowth in p143p53PC12 cells is rather caused by the failure of the p53-dependent regulation of transcription than by the perturbed phosphorylation of endogenous p53. Upon neurite-outgrowth inducing dbcAMP treatment, however, we could detect some phosphorylation signals on serine 46 in wt but not in p143p53PC12 cells (Fig. 3, lanes 3). In addition serine 20 was phosphorylated only upon apoptosis inducing anisomycin treatment, but again only in wtPC12 cells (Fig. 3, lanes 4). Following 6 h of serum withdrawal we detected even more pronounced differences (Fig. 3, lanes 5). While serum starvation only slightly reduced p53 phosphorylation on serine 6 in wtPC12 cells, it was strongly reduced in p143p53PC12 cells. Similarly, while phosphorylation on serine 46 and 392 was slightly induced in wtPC12 cells upon serum starvation, phosphorylation on serine 46 remained undetectable and on serine 392 only weakly detectable in p143p53PC12 cells. Following UV irradiation phosphorylation on serine 6 was significantly reduced in p143p53PC12 cells compared to the wt counterparts. Moreover, UV irradiation induced phosphorylation on serine 392 in wtPC12, but not in p143p53PC12 cells (Fig. 3, lanes 6). In addition, whereas etoposide induced rapid phosphorylation of serine 6 and 15 residues in both cell types, phosphorylation on serine 392 seemed to be a bit slower in p143p53PC12 cells compared to wtPC12 cells. Moreover, phosphorylation of serine 46 was not induced by neither shorter, nor longer etoposide exposures in p143p53PC12 cells (Fig. 3, lanes 7–8).

Expression of V143A Mutant p53 Alters ERK Phosphorylation in PC12 Cells

In response to differentiation inducing agents ERK-1 and -2 are quickly phosphorylated in PC12 cells to transmit the differentiation signal to the nucleus [Yaka et al., 1998]. In order to study the possible role of p53 in NGF-induced ERK activation we analyzed ERK phosphorylation in the p143p53PC12 clone. As Figure 4 shows the total amount of ERK proteins was not affected in p143p53PC12 cells, but general reduction of ERK phosphorylation was observed compared to wtPC12 cells. NGF, dbcAMP and anisomycin slightly increased, while serum starvation and etoposide reduced ERK phosphorylation in p143p53PC12 cells. Since in proliferating, untreated wtPC12 cells ERK activity is relatively high and is hardly affected by various treatments (except serum withdrawal), in order to further analyze the NGF-induced activation of ERKs we kept wtPC12 and p143p53PC12 cells in serum free medium for 6 h to reduce the basal activation of ERKs and then subjected them to NGF treatment for the times indicated in Figure 5. As expected, 6 h of serum starvation efficiently reduced the phosphorylation of ERKs both in wt and transfectant cells (Fig. 5, lanes 1). The time course of ERK phosphorylation upon NGF treatment in p143p53PC12 cells, however, was different than that of the wtPC12 cells. In serum-starved wtPC12 cells NGF-induced ERK

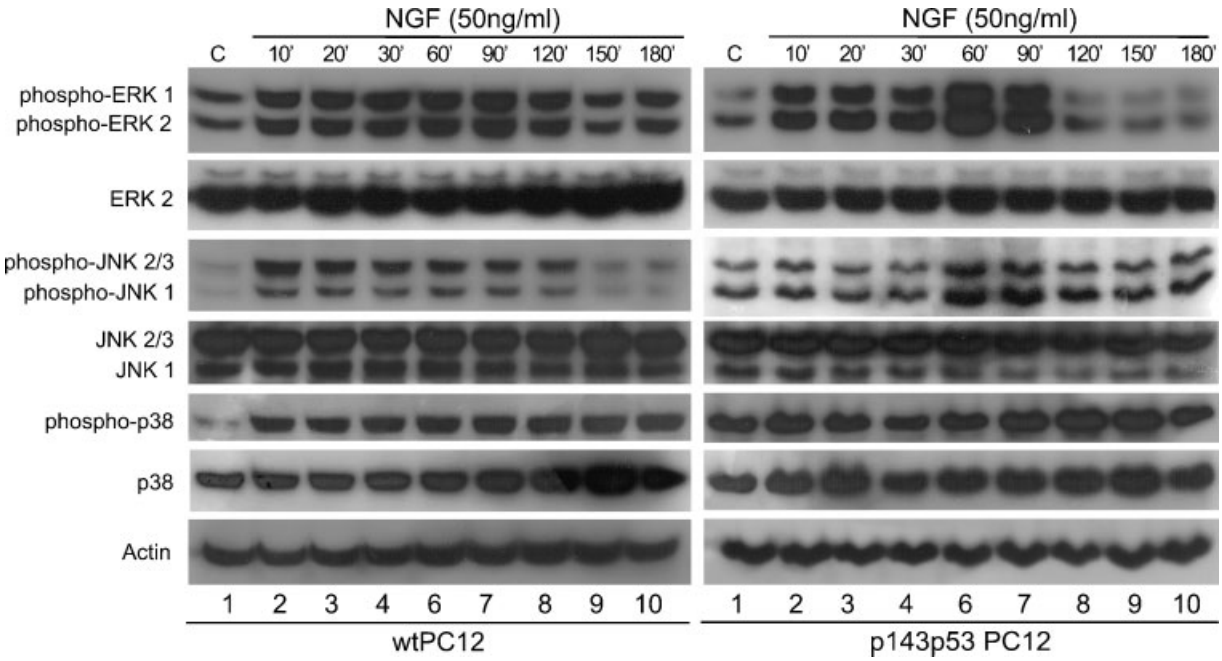

Fig. 5. Time course of phosphorylation of mitogen-activated protein kinases in wtPC12 and p143p53PC12 cells upon NGF treatment. Cells were serum starved for 6 h before treated with NGF (50 ng/ml) at 37°C for the times indicated. Whole cell lysates were subjected to immunoblot analysis of phosphorylated forms of ERK-1, -2, SAPK/JNK-1, -2, -3, and p38 MAP kinases using phosphorylation sites directed antibodies listed in Materials and Methods.

activation lasted for up to 180 min and ERKs were only deactivated after 120 min of the treatment. In contrast, NGF treatment of p143p53PC12 cells for 60 and 90 min caused stronger phosphorylation of ERKs in these cells compared to the appropriate samples of wt counterparts, but ERK phosphorylation abruptly fell back to the basal level after 90 min of treatment. Sustained ERK phosphorylation in NGF-treated PC12 cells was thus made transient by the presence of the dominant inhibitory p53 protein.

Expression of the V143A Mutant p53 Causes Constitutive Activation of Stress-Induced MAP Kinases in PC12 Cells

In order to further characterize the MAPK status of our transfectant PC12 cells upon NGF treatment we analyzed activation of two additional MAPKs, JNK/SAPK and p38. Upon serum starvation both stress-induced MAPKs were dephosphorylated in wtPC12 cells (Fig. 5, lanes 1). NGF treatment caused, as expected, phosphorylation of both JNK/SAPK and p38 within minutes. NGF-induced phosphorylation of JNK/SAPK isoforms on Thr183/Tyr185 was sustained for about 120 min and then it was terminated with a time course similar to the dephosphorylation of ERKs in wtPC12 cells. In contrast, activating phosphorylation of p38 on Thr180/Tyr182 residues by NGF was also induced within 10 min, but p38 remained phosphorylated for up to 180 min. Interestingly, in PC12 cells expressing the V143A mutant p53 both JNK/SAPK and p38 remained phosphorylated 6 h after serum starvation. In addition, we could not observe any response to NGF in p38 phosphorylation. Although JNK/SAPK was also phosphorylated in serum-starved, but untreated p143p53PC12 cells (Fig. 5, lane 1), a delayed phosphorylation of JNK/SAPK could finally be detected at 60–90 min of NGF treatment resembling the time course of NGF-induced ERK phosphorylation (Fig. 5).

DISCUSSION

p53 lies at the heart of the cellular mechanisms responsible for the biological responses to cellular insults including genotoxic stress or growth factor withdrawal. It has an essential role in the halt of cell cycle, induction of DNA repair genes or initiation of intrinsic apoptotic processes. The aim of the present study was to

analyze the role of p53 in differentiation and stress signaling in the PC12 cell system. We found that the expression of a dominant negative p53 mutant had a profound effect on protein phosphorylation cascades mediating these functions.

Considering the complex regulatory machinery of p53 and its crucial role in the life of mammalian cells, it is not surprising that malfunctions of p53 have severe consequences to the cells. While germline mutations of p53 in humans lead to the relatively rare Li–Fraumeni syndrome with development of multiplex tumors early in life [Varley, 2003], somatic mutations of the TP53 gene are much more common and were found in about 50% of human cancers [Hollstein et al., 1991]. To date several thousands of TP53 mutations have been described most of them being loss of function mutations. Certain mutations act in a dominant negative fashion and usually affect the DNA binding domain of p53. Some of them show temperature sensitivity: at physiological temperatures they fail to bind to p53 consensus sequences but at lower, permissive temperatures they behave like their wt counterparts [Friedlander et al., 1996]. Certain temperature sensitive mutants reduce the NGF-induced wt p53 activity by inhibiting its deacetylation at lysine 382 [Vaghefi and Neet, 2004]. In wtPC12 cells biallelic inactivation of wtp53 inhibits NGF-induced differentiation and growth arrest [Eizenberg et al., 1996; Hughes et al., 2000].

Since dominant inhibitory mutants may affect the p53 pathway differently than simple loss of the endogenous protein (see above), to analyze this possibility we transfected wtPC12 cells expressing wtp53 protein with a cDNA coding for the dominant inhibitory (V143A mutant) p53 protein. A stable transfectant strongly expressing the V143A p53 was exposed to a variety of stimuli that induce p53 activation in wt PC12 cells. We found that p143p53PC12 cells, in contrast to wtPC12 cells, did not grow neurites and continued their proliferation in the presence of NGF (Fig. 1). Expression of the V143A p53 protein in PC12 cells efficiently reduced the amount of DNA-bound p53 in nuclear extracts of untreated cells as well as in cells exposed to various pro- or anti-apoptotic p53-inducing treatments (Fig. 2). Since induction of c-Myc DNA-binding was not affected in p143p53PC12 cells (Fig. 2), this effect was restricted to the p53 regulatory circle and was not part of a general depression of transcriptional activity.

Functional p53 regulates, at least in part, its own expression [Deffie et al., 1993]. In addition, p53 is also regulated by post-translational modifications like phosphorylation and acetylation [Brooks and Gu, 2003]. To look for the reason for decreased p53 DNA binding in p143p53PC12 cells, we compared the level and phosphorylation of p53 protein in wt and mutant PC12 cells (Fig. 3) The V143A protein down-regulated the expression of endogenous p53 protein. The basal level of phosphorylation of serines studied in this work (Ser 6, 15, 20, 46, 392) looked very similar in wtPC12 and p143p53PC12 cells indicating that the accessibility of these sites to protein kinases is not affected by the mutation. NGF treatment had very little effect on these phosphorylation sites and the pattern of phosphorylation was very similar in the two cell lines. The lack of differentiation response of p143p53PC12 cells to NGF treatment is thus caused by the reduced level of p53 protein rather than its altered phosphorylation. Decreased phosphorylation was observed in the mutant cell line after certain treatments, at specific sites: phosphorylation of Ser 6, a target of casein kinase 1 δ and ε in response to genotoxic stress [Milczarek et al., 1997] was attenuated in serum-starved and UV-irradiated cells; Ser 392 of the tetramerization domain [Yap et al., 2004] behaved similarly; phosphorylation of Ser 46, target site for ATM [Saito et al., 2002] and homeodomain-interacting protein kinase-2 [D'Orazi et al., 2002] was strongly inhibited in serum-starved and etoposide-treated p143p53PC12 cells. The expression of V143A p53 protein can thus affect differentially the phosphorylation sites in the p53 protein, but the biochemical and biological consequences are not clear.

The Ras/Raf/MEK/ERK pathway plays a critical role in differentiation signaling from the NGF receptor [Szeberenyi and Erhardt, 1994; Chang et al., 2003a]. In this pathway ERK proteins are key players: the extent and kinetics of their phosphorylation determine the biological responses of PC12 cells [Chang et al., 2003b]. Connections between the Ras/Raf/MEK/ERK pathway and p53 have already been demonstrated [Wu, 2004]. Activation of Ras up-regulates $p19^{ARF}$ leading to p53 stabilization by the inhibition of MDM2 [Lin and

Lowe, 2001]. In addition, the promoter region of p53 contains a binding site for ETS1 and 2 [Venanzoni et al., 1996], transcription factors that regulate transcription in response to activated Ras, and ETS1 is required for p53 transcriptional activity to induce apoptosis upon UV irradiation [Xu et al., 2002]. Our studies on ERK phoshporylation indicate that p53 and the ERK pathway are connected in the opposite direction as well. Phosphorylation of ERKs is strongly inhibited in cycling p143p53PC12 cells under various conditions (Fig. 4). Quiescent cells, on the other hand, respond well to NGF treatment, but ERK phosphorylation, while robust, becomes transient in p143p53PC12 cells, compared to the more sustained ERK activation in wtPC12 cells (Fig. 5). The premature termination of ERK phosphorylation in p143p53PC12 cells resembles the kinetics of EGF-induced ERK activation of wtPC12 cells [Qui and Green, 1992; Marshall, 1995], or NGF stimulation of ERKs in PC12 cells with partial inhibition of Ras function [Boglari et al., 1998]: neuritogenesis is blocked under these conditions.

The mechanism of transient activation of the ERK pathway in p143p53PC12 cells by NGF is not known. One possible explanation is an increased protein phosphatase activity prematurely terminating signaling through this pathway. Several protein phosphatases are implicated in the dephosphorylation of ERKs (MKP-1, -3, -6, VHR1, PAC1, DUSP-5) most of them being transcriptionally up-regulated by p53 [Li et al., 2003; Wu, 2004]. Expression of some members of the Cdc25 family of phosphatases is, however, down-regulated by p53 [Krause et al., 2001; St Clair et al., 2004]. Impaired p53 function in p143p53PC12 cells may thus increase a Cdc25 activity that in turn would dephosphorylate Raf-1 leading to premature cessation of signaling through the ERK-pathway, and block of neuritogenesis in p143p53PC12 cells.

Besides the Ras/Raf/MEK/ERK pathway p53 is also implicated in the regulation of stress signaling. Both p38 and SAPK/JNK phosphorylate p53 on serine residues [Wu, 2004]. Although most of these phosphorylations stabilize p53 and trigger its transcriptional activity, p38-induced phosphorylation of p53 on serine 15 prevents the nuclear translocation of p53 inhibiting its transcriptional regulatory role [Chouinard et al., 2002]. In p143p53PC12 cells constitutive activation of both p38 and SAPK/JNK was observed (Fig. 5), that, again, may be caused by the malfunction of a p53-regulated phosphoprotein phosphatase.

Our results thus suggest that several MAPK pathways, while being important regulators of the p53 protein, are also targets of it, giving a further layer of sophistication to the complex p53 regulatory system.

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