A nuclear protein tyrosine phosphatase activates p53 and induces caspase-1-dependent apoptosis

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Abstract PTP-S2/TC45 is a nuclear protein tyrosine phosphatase, which induces p53-dependent apoptosis. Here we show that the p53 protein level increased in MCF-7 cells in response to PTP-S2 overexpression. PTP-S2-induced p53 protein was transcriptionally active and it could activate caspase-1 gene expression from endogenous as well as ectopic promoter. Coexpression of an active site mutant of procaspase-1 strongly inhibited PTP-S2-induced apoptosis. Mutant procaspase-1 also inhibited apoptosis induced by p53 overexpression or doxorubicin treatment, which induce caspase-1 gene expression. In contrast, apoptosis induced by staurosporine or cycloheximide, which do not increase caspase-1 gene expression, was not affected by mutant procaspase-1. These results suggest that caspase-1 may be one of the mediators of p53-dependent apoptosis in human cells.

Key words: Caspase-1; p53; Protein tyrosine phosphatase; Apoptosis

1. Introduction

The tumor suppressor protein p53 plays an important role in mediating a response to stress such as that induced by DNA damage or hyperproliferative signals resulting in either growth arrest or apoptosis [1]. The apoptosis inducing activity of p53 is a major contributor to its tumor suppressor function [2]. p53 protein has a high turnover rate and is maintained at very low levels under normal conditions. Upon exposure to various stimuli p53 is activated through post-translational modifications that increase both its stability and activity [3]. Activated p53 is a sequence-specific DNA-binding protein, which functions as a transcription factor. It is generally believed that many genes regulated transcriptionally by p53 mediate p53-dependent apoptosis. These p53 targets include BAX, p53AIP, Noxa, PUMA, PIGs, Apaf-1, PIDD, Fas, Killer/DR5 etc. [4]. Recently, we have shown that human caspase-1 is a direct transcriptional target of p53 [5].

PTP-S2/TC45 is a ubiquitously expressed nuclear protein tyrosine phosphatase, which binds to DNA non-specifically [6]. Mitogenic stimulation of a variety of cells increases the PTP-S2 mRNA level transiently in G1 phase suggesting a role for this phosphatase in a process related to cell proliferation [6,8,9]. Overexpression of PTP-S2 at moderate levels promotes progression of cells through G1 to S phase in HeLa cells [10,11]. PTP-S2 overexpression at high levels by transient transfections induces apoptosis in p53-positive cells but not in p53-negative cells [12]. Overexpression of PTP-S4/TC48 (a splice variant of PTP-S2 which differs from PTP-S2 in subcellular location, substrate specificity and other properties) induces much less apoptosis in p53-positive cells. PTP-S2-induced apoptosis is inhibited by coexpression of His 273 mutant of p53 [12]. Since mutant p53 (273 His) carries a mutation in sequence-specific DNA-binding region, these observations suggest that PTP-S2-induced apoptosis requires transcriptional activation function of p53 dependent on sequence-specific DNA-binding. However, the role of p53 in PTP-S2-induced apoptosis is not known.

Here we have analyzed the role of p53 and caspase-1 in PTP-S2-induced apoptosis. Our studies show that PTP-S2 expression leads to an increase in the level and activity of p53 protein. PTP-S2 expression results in an increase in caspase-1 mRNA level and caspase-1 promoter activity in a p53-dependent manner. Our results suggest that PTP-S2-induced caspase-1 gene transcription, mediated by p53, contributes to apoptosis in human tumor cell lines. In addition, we show that caspase-1 contributes to p53-induced apoptosis.

2. Materials and methods

2.1. Expression vectors and antibodies

Human procaspase-1 cDNA (α-form) was amplified by reverse transcription polymerase chain reaction (RT-PCR) using RNA from HeLa cells. It was cloned in pCB6$^+$ expression vector in the *Eco*RI site. Mutant procaspase-1 was prepared by replacing Cys 285 (TGC) with Ala (GCC) by PCR-based site-directed mutagenesis. The nucleotide sequence of the mutant and wild-type procaspase-1 cDNA was confirmed by automated sequencing. Plasmids for expressing PTP-S2 (induced by removal of tetracycline) have been described previously [10]. Plasmids expressing p73DD and p53DD, specific inhibitors of p73 and p53, respectively [13], were kindly provided by Dr. W.G. Kaelin, Harvard Medical School, USA. The antibodies used were: p53 antibody, goat polyclonal from Roche Molecular Biochemicals, Cdk-2 and caspase-1, Santa Cruz Biotechnology. PTP-S2 monoclonal antibody has been described previously [7]. T7 tag antibody was from Novagen.

2.2. Cell culture, transfections and immunofluorescence staining

The cell lines were maintained at 37°C in a CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The transfections were done using Lipofectamine Plus™ reagent (Life Technologies, Inc.) according to the manufacturer's instructions. All the plasmids for transfection were prepared by using

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Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; cmk, chloromethylketone

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Qiagen columns. For immunofluorescence staining, cells grown on coverslips were transfected with the required plasmids, fixed after 24 or 48 h and stained with required antibodies as described earlier [6].

2.3. Reporter plasmids and reporter assays

The reporter plasmid pCAT-ICE-WT which contains human caspase-1 promoter from −182 to +42 relative to the transcription start site cloned upstream of chloramphenicol acetyltransferase (CAT) reporter gene has been described [14]. The reporter plasmid pCAT-ICE-MT was derived from pCAT-ICE-WT by mutating the p53-responsive site [5]. Cells grown in 60 mm dishes were transfected with 500 ng of pCAT-ICE-WT (or pCAT-ICE-MT), 200 ng of pCMV-SPORT-β-Gal (Life technologies, Inc.) and 750 ng each of pTRE-PTP-S2 and pTet-Off plasmids, or control plasmids. Preparation of lysates and CAT assays were carried out as described [5].

2.4. Apoptosis assay

Quantitative analysis of apoptotic cells was carried out essentially as described previously [12]. Cells grown on coverslips were transfected with the required plasmids and processed for immunofluorescence staining using appropriate antibodies. For caspase-4-induced apoptosis, GFP plasmid was included to visualize transfected cells. Cells were mounted in 90% glycerol containing 1 mg/ml paraphenylenediamine (antifade) and 0.5 μg/ml DAPI (4′,6-diamidino-2-phenylindoledihydrochloride) to stain the DNA. Cells showing immunofluorescence staining were counted and those cells that showed loss of cell volume, loss of refractility and condensed chromatin were scored as apoptotic. At least 200 expressing cells were counted in each coverslip. The data represent mean ± S.D. from at least three independent experiments. Cells not expressing the transfected protein were also counted in each coverslip. Generally 1–3% of non-expressing cells showed apoptosis.

2.5. RT-PCR

Total RNA was isolated using Trizol™ reagent (Life Technologies, Inc.). Semiquantitative RT-PCR was carried out essentially as described previously [5,6]. RNA was reverse-transcribed using reagents from first strand cDNA synthesis kit (Life Technologies, Inc.). The caspase-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were amplified in the same reactions for 40 and 23 cycles, respectively. Primers for amplification of caspase-1 and GAPDH have been described previously [5,6]. Appropriate gene-specific primers were used for amplification of various caspases and other genes.

3. Results and discussion

3.1. Activation of p53 upon overexpression of PTP-S2

PTP-S2 overexpression in MCF-7 cells was achieved by transient transfection of pTet-Off and pTRE-S2 (PTP-S2 cloned in pTRE) plasmids. Twenty-four hours after transfection cells were trypsinized and plated on two plates: one induced (by removal of tetracycline) and the other one uninduced. The cells were harvested after 24 h of induction. PTP-S2 expression upon induction was confirmed by immunoblot using PTP-S2 monoclonal antibody G11 (Fig. 1A). This monoclonal antibody recognizes rat or mouse PTP-S2 protein but not the endogenous human protein [6,7]. The level of p53 protein markedly increased upon expression of PTP-S2 in MCF-7 cells (Fig. 1A). Equal loading of protein on the immunoblot was confirmed by reblotting the membrane with Cdk-2 antibody (Fig. 1A). The half-life of PTP-S2-induced p53 protein was about 2 h as determined by treatment of cells with protein synthesis inhibitor cycloheximide (Fig. 1A). The half-life of p53 protein in uninduced cells could not be determined due to the very low level of this protein. The half-life of p53 in MCF-7 cells has been reported to be about 30 min [15] indicating that PTP-S2 overexpression may be stabilizing the p53 protein. There was no increase in the p53 mRNA upon PTP-S2 overexpression in MCF-7 cells as determined by semi-quantitative RT-PCR analysis (Fig. 1B). These results suggest that induction of p53 protein by PTP-S2 occurred by a post-transcriptional mechanism. Genes that enhance proliferation are known to induce p53 through induction of $p14^{ARF}$ [16], but a PTP-S2-induced increase in p53 protein is likely to be through a $p14^{ARF}$-independent pathway since MCF-7 cells lack $p14^{ARF}$ function due to a deletion in the gene [17].

To analyze whether the PTP-S2-mediated upregulation of endogenous p53 leads to the activation of p53 transcriptional function, we determined the level of p21 mRNA which is a known transcriptional target of p53. For this purpose, we used MCF-7 cells and MCF-7-mp53, a clone of MCF-7 cells expressing His 273 mutant of p53. The MCF-7-mp53 clone was obtained by transfection of MCF-7 cells with His 273 mutant of p53 followed by selection in G418 [5]. This mutant of p53 has been shown to act as dominant inhibitor of wild-type p53 function [18]. Induction of PTP-S2 expression after transient transfection resulted in an increase in p21 mRNA in MCF-7 cells, but not in MCF-7-mp53 cells (Fig. 1C). These results showed that p53 protein induced by PTP-S2 expression is transcriptionally active.

3.2. Induction of caspase-1 gene expression by PTP-S2

Caspase-1 gene expression in human cells is regulated by p53 [5]. Since PTP-S2-induced apoptosis was p53-dependent, we analyzed the expression of caspase-1 in MCF-7 cells in response to PTP-S2 expression. The caspase-1 mRNA level was very low and it increased several fold in MCF-7 cells by induction of PTP-S2 expression as compared with uninduced cells (Fig. 2). There was no increase in caspase-1 mRNA level upon PTP-S2 overexpression in MCF-7-mp53 cells (Fig. 2). These results suggested that the induction of caspase-1 mRNA by PTP-S2 expression was dependent on functional p53 protein. There are five isoforms of human caspase-1, which differ in their apoptotic activities [19]. Using appropriate primers we determined that the pro-apoptotic α-isoform of caspase-1 was upregulated upon PTP-S2 over-

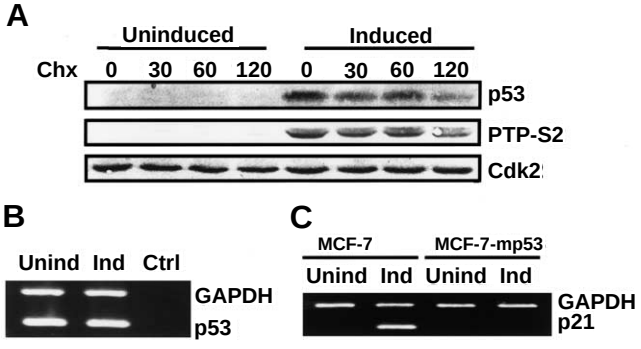
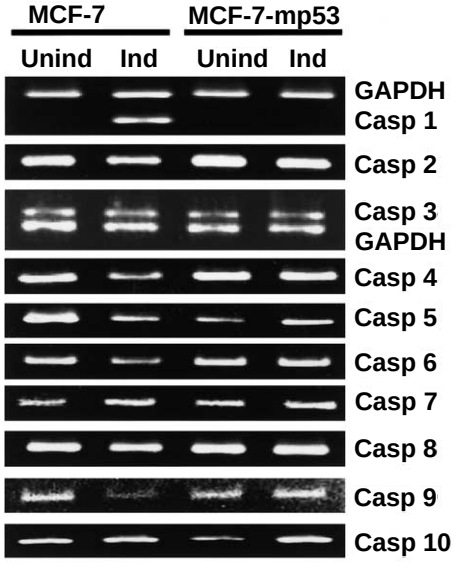


Fig. 1. PTP-S2-induced increase in p53 protein level. A: MCF-7 cells were transfected with PTP-S2 and were induced or uninduced for PTP-S2 expression. The treatment with 25 μg/ml cycloheximide (Chx) was done for 30–120 min. Immunoblotting of total protein was performed using antibodies against PTP-S2, p53, and Cdk-2. B: RT-PCR analysis of total RNA isolated from MCF-7 cells, induced (*ind*) and uninduced (*unind*) for PTP-S2 expression. RT products were used as a template to amplify human p53 and GAPDH mRNAs using gene-specific primers for human p53 and GAPDH. An ethidium bromide-stained agarose gel with indicated PCR products is shown. C: RT-PCR analysis of total RNA isolated from MCF-7 and MCF-7-mp53 cells, induced (*ind*) and uninduced (*unind*) for PTP-S2 expression using primers for p21 and GAPDH.

expression and other forms were not induced (data not shown). Expression of p53 also induced only the α-isoform of caspase-1 [5]. We analyzed the level of mRNA of some other caspases in response to PTP-S2 overexpression. There was no increase in the level of caspase-2, caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-8, caspase-9 or caspase-10 mRNAs. There was some decrease in the level of caspase-4, caspase-5 and caspase-9 mRNA, which was p53-dependent (Fig. 2). These results suggest that p53-dependent induction of caspase-1 gene expression upon overexpression of PTP-S2 is a very specific effect and not a general effect on caspase gene expression.

Multiple transcriptional targets are believed to be involved in mediating p53-induced apoptosis [3,4,20]. Therefore we analyzed the level of mRNA of some of the known pro-apoptotic genes in response to expression of PTP-S2. Apaf-1 was induced upon PTP-S2 expression in MCF-7 cells but not in MCF-7-mp53 cells (Fig. 2). There was no significant increase in BAX or PUMA mRNA in both MCF-7 and MCF-7-mp53 cells upon PTP-S2 expression although doxorubicin treatment increased the BAX and PUMA mRNA level in MCF-7 cells (Fig. 2).



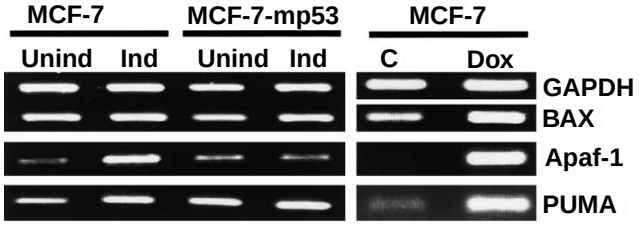


Fig. 2. PTP-S2 induces caspase-1 gene expression in a p53-dependent manner. RT-PCR analysis of total RNA isolated from MCF-7 and MCF-7-mp53 cells, induced (*ind*) and uninduced (*unind*) for PTP-S2 expression after transient transfection. RT products were used as a template to amplify indicated mRNAs using respective gene-specific primers. Ethidium bromide-stained agarose gel with indicated PCR products is shown. Doxorubicin (Dox)-treated and untreated (C) controls are shown for BAX, Apaf-1 and PUMA.

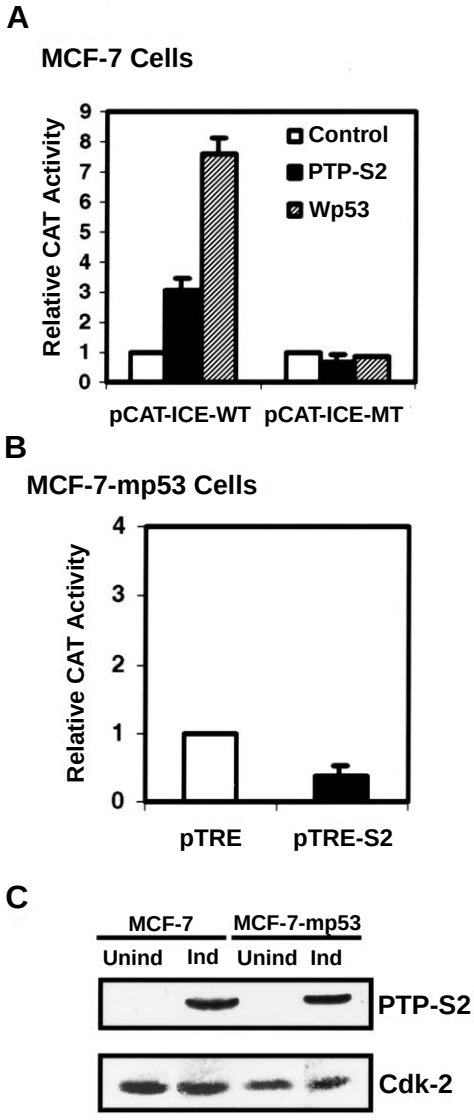


Fig. 3. Transactivation of caspase-1 promoter by PTP-S2 overexpression. A: Effect of disruption of the p53-binding site in caspase-1 promoter on its transactivation by PTP-S2. The wild-type promoter–reporter (pCAT-ICE-WT) or mutant promoter–reporter plasmid (pCAT-ICE-MT) was cotransfected with pCMV-SPORT-β-Gal and PTP-S2 (pTRE-S2), wild-type p53 (Wp53) or control plasmids in MCF-7 cells. CAT activities relative to control (pTRE) are shown ($n = 3$). B: pCMV-SPORT-β-Gal plasmid and pCAT-ICE-WT plasmids were cotransfected, along with PTP-S2 (*pTRE-S2*) or control (*pTRE*) plasmids in MCF-7-mp53 cells. CAT activities relative to control (*pTRE*) are shown ($n = 3$). C: Expression of PTP-S2 in MCF-7 and MCF-7-mp53 cells. Immunoblotting was performed with total proteins isolated from MCF-7 and MCF-7-mp53 cells, induced (*ind*) and uninduced (*unind*) for PTP-S2 expression using antibodies against PTP-S2, and Cdk-2.

3.3. *PTP-S2-induced transactivation of caspase-1 promoter requires p53*

We tested whether PTP-S2-induced accumulation of endogenous p53 leads to transactivation of human caspase-1 promoter–reporter construct. To this end, we carried out CAT reporter assays in transiently transfected MCF-7 cells. As shown in Fig. 3A, PTP-S2 (and p53) activated the reporter construct having a wild-type p53-binding site (pCAT-ICE-WT) but not the reporter construct where the p53-binding

site was abolished by mutation (pCAT-ICE-MT). PTP-S2 expression was unable to transactivate pCAT-ICE-WT construct in MCF-7-mp53 cells; instead, there was some decrease in promoter activity upon PTP-S2 expression (Fig. 3B). This difference in activation of pCAT-ICE-WT by PTP-S2 in the two cell lines was not due to variation in the levels of exogenously expressed PTP-S2 protein (Fig. 3C). These results showed that PTP-S2-induced transcriptional activation of human caspase-1 promoter was through a p53-binding site and was dependent on the functional p53 protein in the cell.

Previously, it has been shown that PTP-S2 expression does not induce apoptosis in HeLa cells [12], which are functionally p53-negative. In these cells, caspase-1 mRNA was not induced by PTP-S2 (data not shown). For most of our experiments described here we have used MCF-7 and MCF-7-mp53 cell lines. Therefore, we analyzed the extent of apoptosis in MCF-7-mp53 cells upon PTP-S2 expression. PTP-S2-induced apoptosis was drastically reduced in MCF-7-mp53 cells in which caspase-1 mRNA was not induced by PTP-S2 (data not shown).

The p53 homologue, p73, is an inducer of apoptosis, which activates transcription of many p53-responsive genes such as p21, mdm2, BAX and GADD45 [21]. To address the role of p73 in PTP-S2-induced apoptosis we used p73DD and p53DD, which are specific inhibitors of p73 and p53, respectively [13]. PTP-S2-induced apoptosis was inhibited by cotransfection with p53DD but not by p73DD (Fig. 4A). This was not due to a lower level of expression of p73DD (Fig. 4B). This experiment showed that p73 is not required for PTP-S2-induced apoptosis.

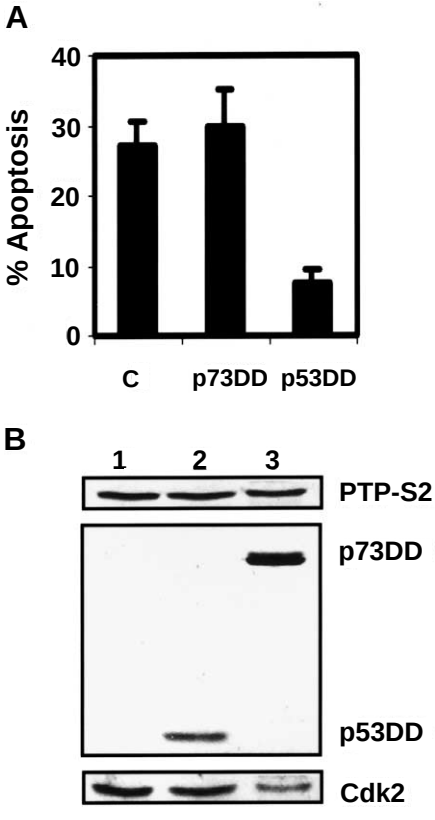


Fig. 4. PTP-S2-induced apoptosis requires p53 but not p73. A: Effect of cotransfection of p73DD, p53DD or control plasmid (C) on PTP-S2-induced apoptosis in MCF-7 cells. B: Immunoblot showing expression of p73DD and p53DD using T7 epitope antibody.

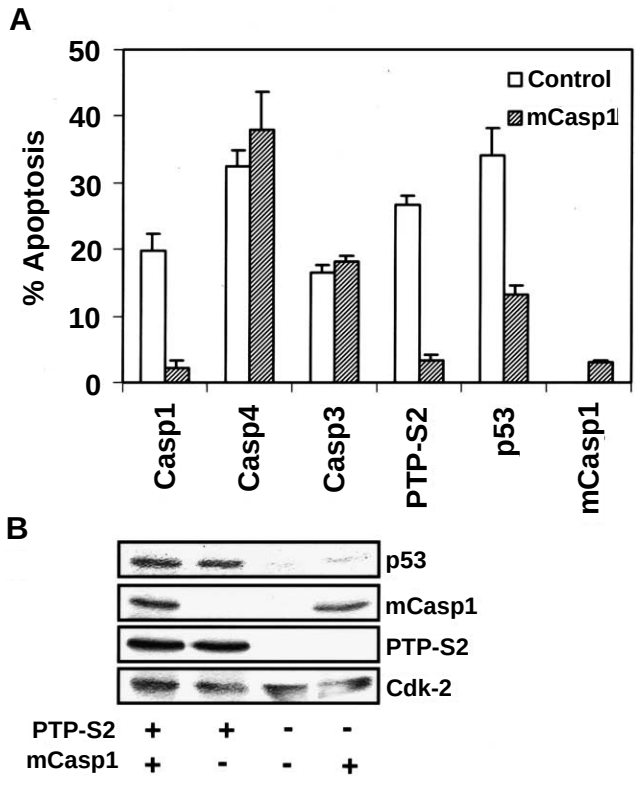


Fig. 5. Caspase-1 is required for PTP-S2-induced and p53-induced apoptosis. A: Effect of coexpression of mutant caspase-1 (mCasp-1) or control plasmid on apoptosis induced by caspase-1, caspase-3, caspase-4, PTP-S2 or p53 in MCF-7 cells. Cells were fixed and processed for immunofluorescence after 48 h (PTP-S2, caspase-1, caspase-3 and caspase-4) or 24 h (p53) of transfection. The total amount of DNA transfected was kept constant. B: Expression of mutant caspase-1 does not effect induction of p53 by PTP-S2. MCF-7 cells were transfected with PTP-S2 plasmid with or without mutant caspase-1. After 48 h the cell lysates were subjected to immunoblotting with indicated antibodies.

3.4. Requirement of caspase-1 for p53-dependent apoptosis

Since caspase-1 gene expression was increased upon PTP-S2 expression in a p53-dependent manner we analyzed the role of caspase-1 in PTP-S2-induced as well as p53-induced apoptosis. For this purpose a catalytically inactive mutant of human procaspase-1 was prepared by replacing active site cysteine with alanine. This Ala 285 mutant of procaspase-1 inhibited caspase-1-induced apoptosis but not caspase-3- or caspase-4-induced apoptosis suggesting that it is a specific inhibitor (Fig. 5A). PTP-S2-induced apoptosis in MCF-7 cells was strongly inhibited by coexpression of mutant procaspase-1 and apoptosis induced by transfection of MCF-7 cells by normal p53 was partially (over 60%) inhibited by mutant procaspase-1 (Fig. 5A). Inhibition of PTP-S2-induced apoptosis by mutant procaspase-1 was not due to decrease in p53 protein induction (Fig. 5B). PTP-S2 as well as p53-induced apoptosis in A549 human lung cancer cell line was also inhibited by mutant procaspase-1 (data not shown). We found that apoptosis induced by doxorubicin treatment of MCF-7 cells was partially inhibited by mutant procaspase-1 (Fig. 6A). Apoptosis in-

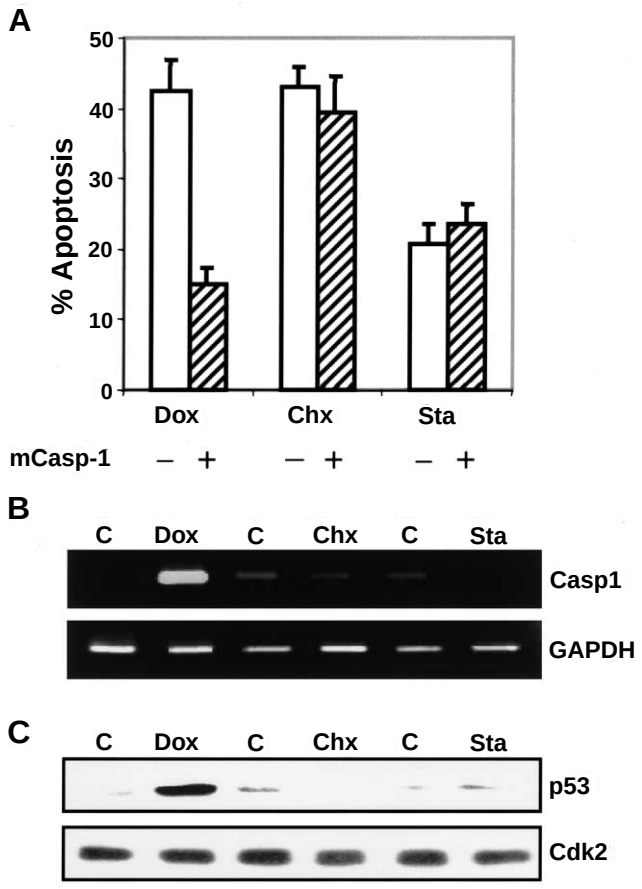


Fig. 6. Effect of expression of mutant caspase-1 on apoptosis induced by treatment with doxorubicin (Dox), cycloheximide (Chx) or staurosporine (Sta). A: MCF-7 cells were transfected with mutant caspase-1 and 24 h after transfection, the cells were treated with doxorubicin (0.5 μg/ml, 24 h), cycloheximide (100 μg/ml, 16 h) or staurosporine (0.1 μM, 12 h). Cells were fixed and stained with caspase-1 antibody to visualize expression of transfected mutant caspase-1. The percentage of cells showing apoptosis in mutant caspase-1 expressing and non-expressing cells was determined. mCasp-1, mutant caspase-1. Panel B shows the effect of treatment of MCF-7 cells with doxorubicin, cycloheximide or staurosporine on the caspase-1 mRNA level. Panel C shows the p53 protein level in cells treated with doxorubicin, cycloheximide or staurosporine.

duced by treatment of MCF-7 cells with cycloheximide or staurosporine, which did not induce caspase-1 gene expression (Fig. 6B) or p53 protein (Fig. 6C), was not inhibited by mutant procaspase-1 (Fig. 6A). Thus, mutant procaspase-1 inhibited apoptosis induced by those agents which also induced caspase-1 gene expression.

These results suggest that caspase-1 (or a related caspase inhibited by mutant caspase-1) plays an important role in mediating p53-dependent apoptosis. It is generally believed that many genes induced transcriptionally contribute towards p53-dependent apoptosis and no single gene is absolutely essential. Therefore nearly complete inhibition of PTP-S2-induced apoptosis by mutant caspase-1 is somewhat unexpected. Although other known transcriptional targets of p53 such as Apaf-1 may be induced by PTP-S2 expression, caspase-1 seems to play a predominant role in PTP-S2-induced apoptosis. Several studies have implicated the involvement of caspase-3 in p53-mediated apoptosis. In our studies both PTP-S2 as well as p53 induced apoptosis in MCF-7 cells, which lack functional caspase-3 due to a deletion in the gene [22], implying that caspase-3 is not essential for p53-mediated apoptosis.

A functional p53-binding site has been identified in human caspase-1 promoter [5]. No putative p53-binding site could be identified in the 5′ regulatory sequences or introns of murine caspase-1 gene. These observations indicate that in murine cells caspase-1 gene expression may not be regulated by p53. Apoptosis induced by overexpression of p53 in NIH 3T3 cells was not inhibited by caspase-1 family inhibitor YVAD-chloromethylketone (cmk) but was inhibited by caspase-3 inhibitor DEVD-cmk (70% inhibition). Thymocytes from caspase-1 knockout mice are sensitive to apoptosis induced by ionizing radiation, which is p53-dependent [23]. Although cell type-specific differences in apoptosis are possible it is likely that in murine cells caspase-1 may not be a mediator of p53-dependent apoptosis. Our results presented here suggest that transcriptional activation of caspase-1 contributes towards p53-dependent apoptosis in human cells.

In conclusion our results show that overexpression of PTP-S2 increases the p53 protein level and activity resulting in increased caspase-1 gene expression. Caspase-1 is required for PTP-S2-induced and p53-dependent apoptosis. On the basis of these results we suggest that caspase-1 may be one of the mediators of p53-dependent apoptosis.

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