Chapter 3

ROLE OF p53 IN CASPASE-1 GENE REGULATION BY PTP-S2
3.1 INTRODUCTION

All cells are constantly exposed to environmental cues that signal cell survival or cell death. The regulated tyrosine phosphorylation of cellular proteins is a major control mechanism for diverse cellular processes like cell growth, proliferation, metabolism, differentiation, migration, survival and cell death. The level of protein tyrosine phosphorylation in a cell is kept in control by reversible tyrosine phosphorylation by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Some protein tyrosine kinases such as c-Abl, ARG, and AATYK and a few protein tyrosine phosphatases such as LAR and PTP-S2 have been implicated in the induction of apoptosis (Yuan et al., 1994; Gaozza et al., 1997; Collins et al., 1994; Weng et al., 1998; Radha et al., 1999).

Apoptosis is an evolutionarily conserved form of cell suicide by which an organism eliminates unwanted cells. Apoptosis (programmed cell death) plays important roles in many normal processes, including fetal development, defense processes, immune responses, and adult tissue homeostasis. Apoptosis is distinguished from necrosis by characteristic chromatin condensation, nuclear fragmentation, cell shrinkage and plasma membrane blebbing, whereby cells break into small, membrane-surrounded fragments (apoptotic bodies) that are phagocytosed without inciting an inflammatory response (Wyllie, 1997). Defects in the physiological pathways for apoptosis contribute to many diseases. Although cell death occurs physiologically in self-renewing tissues such as the skin, gut and bone marrow, irreversible loss of cells in post-mitotic cells of the brain and heart can be catastrophic. Conversely, a failure of normal cell turnover in self-renewing tissues results in cell accumulation (cancer, hyperplasia). Deregulated apoptosis mechanisms have been experimentally implicated in AIDS, allograft rejection, Alzheimer’s disease, autoimmunity (lupus, type-I diabetes, rheumatoid arthritis), cancer, heart failure, infectious diseases, inflammation, osteoporosis, Parkinson’s disease, restenosis, stroke and trauma (Fisher, 1994; Thompson, 1995; Los et al., 1997).

3.1.1 Death by a thousand cuts

Cysteine proteases called caspases mediate apoptosis (Thornberry and Lazebnik, 1998; Cryns and Yuan, 1998). This family of intracellular proteases cleave their
substrates after aspartic acid residues. Caspases are produced as inactive zymogens, but can become activated by proteolytic processing at conserved Asp residues. Because caspases cleave their substrates at Asp residues and are also activated by proteolytic processing at Asp residues, these proteases can collaborate in proteolytic cascades, where caspases activate themselves and each other. Caspases are often categorized as either upstream ‘initiator’ caspases or downstream ‘effector’ proteases (Salvesen and Dixit, 1997). The zymogen forms of upstream initiator caspases have long N-terminal pro-domains that function as protein interaction modules, by associating with proteins that trigger caspase activation. By contrast, downstream effector caspases contain short N-terminal prodomains, and are largely dependent on upstream caspases for their proteolytic processing and activation. Twelve caspases have been identified thus far in humans.

3.1.2 Caspase-1 (Interleukin 1-β converting enzyme)
Caspase-1 was originally identified as interleukin 1-β converting enzyme (ICE), the protease that cleaves the precursor of IL-1β into active cytokine (Cerretti et al., 1992). IL-1β is a multifunctional cytokine that has been implicated in pathogenesis of several acute and chronic inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, septic shock and other physiological situations including wound healing and growth of certain leukemias (Dinarello, 1991; DiGiouine and Duff, 1990). In addition, caspase-1 has been shown to process the cytokine precursors of IL-16 and IL-18 (Ghayur et al., 1997; Gu et al., 1997; Fantuzzi et al., 1998).

Since the demonstration that Ced gene of C. elegans encodes a protein similar to mammalian caspase-1 (Yuan et al., 1993) and that overexpression of both genes promotes apoptosis (Miura et al., 1993; Los et al., 1995), caspase-1 has been a subject of intensive research. Caspase-1 (ICE) is one of the few caspases with characterized activities both in cleavage activation of proinflammatory cytokines and in apoptosis (Cohen, 1997; Boudreau et al., 1995; Chin et al., 1997; Gu et al., 1997).

Cerretti and coworkers demonstrated that human caspase-1 gene consists of 10 exons spanning at least 10.6kb (Cerretti et al., 1994). They found a single
transcription start site approximately 33 bp upstream of the initiator Met codon. They mapped the protease gene to 11q23, a site frequently involved in rearrangements in human cancers, including a number of leukemias and lymphomas. The caspase-1 gene has been shown to be downregulated in colonic adenocarcinomas and silenced via DNA methylation in human renal cancer cells (Jarry et al., 1999; Ueki et al., 2001). These findings support the concept that disruption of caspase-1 mediated apoptotic pathways may be involved in tumor formation. There are five human ICE mRNA isoforms, namely \( \alpha, \beta, \gamma, \delta \) and \( \varepsilon \) generated by alternative splicing involving exons 2-7 of the ICE gene, which differ in their apoptotic activity. The isoforms \( \alpha, \beta \) and \( \gamma \) can induce apoptosis upon overexpression but isoforms \( \delta \) and \( \varepsilon \) were unable to induce apoptosis. In addition ICE-\( \varepsilon \) can bind to the p20 subunit of ICE to form an inactive ICE complex, thereby acting as a dominant inhibitor of ICE activity (Alnemri et al., 1995).

Caspase-1 is synthesized as a proenzyme of 45 kDa which undergoes proteolytic cleavage at Asp residues 103, 119, 297 and 316 to produce an active enzyme (Walker et al., 1994; Wilson et al., 1994). The active enzyme is heterotetramer composed of two subunits of 20 and 10 kDa, both of which are required for catalytic activity. Thornberry et al., (1992) demonstrated that caspase-1 is a unique cysteine protease and they described the design of potent peptide aldehyde inhibitors. The N-terminal domain of caspase-1 contains a caspase recruiting domain (CARD), a conserved sequence motif of about 90 amino acid residues. The CARD module mediates the interaction between a number of large prodomain caspases and their corresponding upstream activator adapters, the prototypical examples include APAF-1 and caspase-9 (Zou et al., 1997; 1999; Day et al., 1999; Qin et al., 1999). A serine/threonine kinase RIP2/CARDIAK/RICK engages caspase-1 through direct protein-protein interaction involving CARD motif present at the C-terminal of RIP2 and within the prodomain of caspase-1, thereby promoting its processing (Thome et al., 1998). Similarly, IPAF (ICE-protease activating factor) associates directly and specifically with CARD module of procaspase-1 and activates it (Poyet et al., 2001). COP (CARD only protein) and ICEBERG, are small CARD containing molecules that bind to the corresponding CARD motif present in the prodomain of caspase-1, thereby inhibiting the activation of proprocaspase-1 by displacing the upstream activator.
RIP-2 (Lee et al., 2001; Humke et al., 2000). Thus, activation of caspase-1 in cells is controlled by interactions of CARD module containing proteins having opposing effects on activation of caspase-1.

Gene targeting revealed that caspase-1 plays an important role in the regulation of immune responses (Li et al., 1995; Kuida et al., 1995). ICE-deficient mice developed normally and were fertile. They had major defects in production of mature IL-1β, were resistant to the lethal effects of endotoxin, and inhibited pancreatitis linked secretion of proinflammatory cytokines associated with survival benefits. Cells from ICE−/− mice were sensitive to most apoptotic inducers. However, thymocytes from ICE−/− mice showed partial resistance towards CD95-induced apoptosis, neurons were resistant to trophic factor withdrawal-induced apoptosis and showed reduced ischemic brain injury (Los et al., 1999). The prodomain of caspase-1 is shown to enhance Fas-mediated apoptosis, most probably through facilitation of the activation of caspase-8 (Tatsuta et al., 2000). Detjen et al., have shown that interferon-γ inhibits growth of human pancreatic cells via caspase-1 dependent induction of apoptosis (Detjen et al., 2001). Ona et al., have demonstrated the role of caspase-1 in the progression of Huntington disease in mouse model of Huntington’s disease (Ona et al., 1999).

3.1.3 PTP-S2: A Nuclear Protein Tyrosine Phosphatase

The non-transmembrane protein tyrosine phosphatase PTP-S is ubiquitously expressed and generates four isoforms by alternate splicing, of which PTP-S2 and PTP-S4 are the two major forms (Swarup et al., 1991; Reddy and Swarup, 1995). PTP-S2 is a nuclear protein and binds to DNA non-specifically. The last six amino acid residues of PTP-S2 are replaced by thirty-four amino acids in PTP-S4, which shows perinuclear cytoplasmic localization and does not bind to DNA. The amino acid residues at carboxyl terminal of PTP-S2 and PTP-S4 determine their subcellular localization, substrate specificity, enzyme activity and interaction with DNA (Kamatkar et al., 1996). PTP-S2 and PTP-S4 mRNA levels increase in G1 phase after mitogenic stimulation of a wide variety of cells and during liver regeneration after partial hepatectomy (Rajendrakumar et al., 1993; Nambirajan et al., 1995). Overexpression of PTP-S2 at moderate levels causes increased rate of cell
proliferation due to shortening of G1 phase in HeLa cells (Radha et al., 1997; Ganapati et al., 2001). PTP-S2 overexpression at high levels by transient transfections induces apoptosis in p53 positive (MCF-7 and A549) cells but not in p53 negative (HeLa and SW620) cells (Radha et al., 1999). PTP-S2-induced apoptosis is reduced by coexpression of Bcl2 and cmrA as well as by Z-YVAD-cmk (a potent inhibitor of caspase-1 family) and not by Z-DEVD-cmk (a caspase-3 family inhibitor). Coexpression of PTP-S2 with mutant p53 (His273) inhibited PTP-S2-induced apoptosis (Radha et al., 1999). These observations indicate a role for p53 and caspase-1 in PTP-S2-induced apoptosis. The work presented in this chapter describes the results of experiments carried out to understand the role of tumor suppressor p53 and caspase-1 during PTP-S2-induced apoptosis.

3.2 RESULTS

3.2.1 Induction of endogenous p53 protein by PTP-S2 overexpression

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 post-transfection cells were trypsinized and plated on two plates; one induced (by removal of tetracycline) and one uninduced. The cells were harvested twenty-four hours post-induction. PTP-S2 expression upon induction was confirmed by Western blot using PTP-S2 monoclonal antibody as shown in Fig 3.1A. The level of p53 protein was markedly increased upon expression of PTP-S2 in MCF-7 cells (Fig 3.1A). The equal loading of protein on immunoblot was confirmed by reblotting the membrane with Cdk-2 antibody (Fig 3.1A). Since the transfection efficiency in these experiments ranged between 25-30% (determined by immunofluorescence with PTP-S2 antibody), the actual degree of induction of p53 protein is higher than one detected here by immunoblots. Since p53 is transcription factor and its nuclear localization is crucial for its biological function, we determined the sub-cellular location of p53 induced after PTP-S2 overexpression. MCF-7 cells grown on coverslips transiently transfected with PTP-S2 and twenty-four hours post-induction cells were fixed and processed for immunofluorescence. The cells were then permeablized using 0.5% Triton X-100 and 0.05% Tween-20 for six minutes at room temperature followed by blocking with 2% BSA. The cells were then incubated with anti-PTP-S2 (a mouse monoclonal) and anti-p53 (goat polyclonal) antibodies over-
Figure 3.1 PTP-S2-induced increase in p53 protein level. Panel A, MCF-7 cells were transfected with PTP-S2 and were induced (ind) or uninduced (unind) for PTP-S2 expression. Western blotting of total protein was performed using antibodies against PTP-S2, p53, and Cdk-2. Panel B, MCF-7 cells transiently transfected with PTP-S2 were fixed and stained using anti-PTP-S2 and anti-p53 antibodies. The secondary antibodies were anti-mouse-AMCA and anti-goat-FITC against PTP-S2 and p53 primary antibodies respectively. Panel C, 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.
night at 4°C followed by anti-mouse-AMCA conjugated and anti-goat-FITC conjugated antibodies sequentially. PTP-S2 has been shown to localize to nucleus with a prominent nucleolar staining (Kamatkar et al., 1996). As expected PTP-S2 showed nuclear localization with intense nucleolar staining whereas p53 showed diffuse overall nuclear staining and no significant staining of nucleoli (Fig 3.1B). PTP-S2 expressing cells showed increased nuclear staining with anti-p53 antibody (Fig 3.1B). This marked difference in the staining pattern of PTP-S2 and p53 rules out the cross reactivity of secondary antibodies and indicates the specificity of immunostaining. In addition the untransfected cells did not show any increase in p53 staining indicating that, this effect is not due to the removal of tetracycline itself. This immunofluorescence data corroborates the western blot shown in Fig 3.1A. There was no increase in the p53 mRNA upon PTP-S2 overexpression on MCF-7 cells as determined by semi-quantitative RT-PCR analysis (Fig 3.1C). These results suggest that induction of p53 protein by PTP-S2 occurred by a post-transcriptional mechanism.

3.2.2 PTP-S2-induced upregulation of p53 protein increases its transactivation function

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-mp53, a clone of MCF-7 cells expressing mutant p53 (His 273). The MCF-mp53 clone was obtained by transfection of MCF-7 cells with His 273 mutant of p53 followed by selection in G418. This mutant of p53 has been shown to act as dominant inhibitor of wild-type p53 function (Aurelio et al., 2000). These cells were transfected with PTP-S2 plasmids (pTet-Off and pTRE-S2) and 24 hours after transfection the cells were trypsinized and plated on two plates. One of these plates was induced for PTP-S2 expression for 24 hours. RNA was then isolated and p21 mRNA levels were determined by RT-PCR as described in section 2.2.27. Induction of PTP-S2 expression after transient transfection resulted in an increase in p21 mRNA in MCF-7 cells whereas no increase in p21 mRNA was observed in MCF-mp53 cells (Fig 3.2A). These results showed that p53 protein induced by PTP-S2 expression is transcriptionally active.
Figure 3.2 PTP-S2-induced increase in p21 and caspase-1 gene expression in a p53-dependent manner. Panel A, RT-PCR analysis of total RNA isolated from MCF-7, and MCF-mp53 cells, untransfected (U), induced (ind) and uninduced (unind) for PTP-S2 expression after transient transfection. RT products were used as a template to amplify human p21, Bax, caspase-1 and GAPDH mRNAs using respective gene-specific primers. Ethidium bromide-stained agarose gel with indicated PCR products is shown. Panel B, schematic representation of splice variants of human caspase-1. The positions of primers used for amplifying caspase-1 are indicated by arrows. Panel C, RT-PCR analysis for caspase-1 expression using primers C1P4 and C1P5. A PCR product of 451 is produced from α-isoform. β-isoform would give a PCR product of 388 bp where as γ - and δ-isoforms would give a PCR product of 172 bp.
3.2.3 Induction of Caspase-1 gene expression by PTP-S2

Since inhibitor studies had indicated a possible role of caspase-1 in PTP-S2-induced apoptosis, we analyzed the expression of caspase-1 in these cells. The caspase-1 mRNA level increased several fold in MCF-7 cells by induction of PTP-S2 expression as compared with uninduced cells (Fig 3.2A). There was no increase in caspase-1 mRNA level upon PTP-S2 overexpression in MCF-mp53 cells (Fig 3.2A). These results suggested that the induction of caspase-1 mRNA by PTP-S2 expression was dependent on transcriptional activation of p53. There are five isoforms of human caspase-1, which differ in their apoptotic activities as shown in Fig 3.2B (Alnemri et al., 1995). Using appropriate primers we determined that pro-apoptotic α-isoform of caspase-1 was upregulated upon PTP-S2 overexpression (Fig 3.2C) and β, γ and δ forms were not induced. These results show that PTP-S2 overexpression can induce expression of caspase-1 gene in MCF-7 cells in a p53-dependent manner. There was no significant increase in BAX mRNA in both MCF-7 and MCF-mp53 cells (Fig 3.2A) with expression of PTP-S2.

3.2.4 p53-dependent expression of human caspase-1

The level of caspase-1 mRNA was determined by RT-PCR analysis in response to transient overexpression of human wild-type p53 in MCF-7 cells. Caspase-1 mRNA level increased several folds by overexpression of p53 as compared with the control-transfected cells or untransfected cells (Fig. 3.3A). This increase in caspase-1 mRNA level was not the result of induction of apoptosis by p53 since treatment of MCF-7 cells with other apoptosis inducing agents, staurosporine and cycloheximide did not increase caspase-1 mRNA level (Fig. 3.3 B). Treatment with staurosporine in fact decreased the level of caspase-1 mRNA. Using another set of primers, we found that the α form, which is proapoptotic, was induced by p53 and β, γ and δ forms were not induced (Fig 3.3C). By using appropriate primers we found that the ε-isoform was also not induced (data not shown).

3.2.5 Human caspase-1 promoter has potential p53-binding sites

The sequence of 5' flanking region of human caspase-1 (Accession No. L27475) was downloaded from EMBL/Genbank library (Cerretti et al., 1994). The 1480 bp
Figure 3.3 p53-dependent expression of caspase-1. Panel A, RT-PCR analysis of total RNA from MCF-7 cells transfected with wild-type p53 (Wp53), control (C), and untransfected (U) cells using primers for human caspase-1 and GAPDH. Upper panel shows an ethidium bromide-stained agarose gel of PCR products as indicated, and lower panel shows a southern blot for caspase-1. Panel B, effect of cycloheximide (Chx) and staurosporine (Sta) on caspase-1 mRNA levels in MCF-7 cells. An ethidium bromide-stained agarose gel of PCR products is shown as indicated. Panel C, RT-PCR analysis for caspase-1 expression using primers C1P4 and C1P5. A PCR product of 451 is produced from α-isoform. β-isoform would give a PCR product of 388 bp where as γ- and δ-isoforms would give a PCR product of 172 bp. M, molecular weight markers.
Figure 3.4 *Putative p53-binding sites in human caspase-1 promoter.*

*Panel A,* diagrammatic representation of promoter region of caspase-1. The nucleotide positions of the binding-sites of indicated proteins relative to transcription start site are shown. ATG represents the translational start site. *Panel B,* sequence of the fragment used for CAT constructs. The p53-binding site (-85 to -66) is boxed and p53-binding core sequences are shown in bold. The potential SP1-binding sites are underlined with SP1-binding core sequences in bold. The IRF-binding site and Initiator element is overlined. The *arrow* indicates the transcriptional start site and initiator Met codon is in bold and italicized. The CAAT box is indicated and is in bold. Three other potential p53-binding core sequences are shown in bold.
Figure 3.5 Transactivation of caspase-1 promoter by wild-type p53. pCMV.SPORT-βGal plasmid and pCAT reporter plasmids were cotransfected, along with wild-type p53 (Wp53) or mutant p53 (Mp53) or control (C) plasmids. Panel A, shows a representative thin layer chromatogram of CAT assay with pCAT-ICE-WT (lanes 1-3), and pCAT-Basic reporter (lanes 4-6) and p53 plasmids as indicated in HeLa cells. Untransfected control (U), lane 7. Panel B and C, CAT activities after normalizing with β-Gal relative to control are shown (n=4). The ratio of p53 to reporter plasmid was 1:1 in panel B and 2:1 in panel C.
Figure 3.6 Identification of functional p53-binding site in caspase-1 promoter. Panel A, Schematic representation of caspase-1 promoter-reporter constructs, pCAT-ICE-WT, pCAT-ICE-MT and pCAT-ICE-DEL. The nucleotide sequence of putative p53-binding site of human caspase-1 promoter is shown in the box. Panel B, effect of disruption of the p53-binding site in caspase-1 promoter on transactivation by wild-type p53. The ratio of p53 to reporter plasmid was 2:1 (n=3).
region (-182 to +42) of caspase-1 promoter. In addition, 75 bp of human caspase-1 promoter upstream of transcription start site are sufficient for basal expression of caspase-1 gene.

3.2.8 p53 binds to putative p53-binding site in human caspase-1 promoter
In order to determine whether p53 binds to the putative p53-binding site in human caspase-1 promoter, we carried out electrophoretic mobility shift assays using a synthetic oligonucleotide corresponding to this site (Fig. 3.7A). Binding to this oligonucleotide was seen with nuclear extract prepared from MCF-7 cells treated with doxorubicin, which is known to increase p53 protein level (Fig. 3.7B, lane 2). This binding was competed out with 50-fold excess of unlabeled self-oligonucleotide and also with a consensus p53-binding oligonucleotide but not with a mutated oligonucleotide in which the p53-binding core sequence CATG was mutated to AA TT (Fig. 3.7A, B, lanes 3-5). A polyclonal antibody to p53 immunodepleted the shifted band (Fig. 3.7B, lane 6). These results suggest that the binding to this oligonucleotide corresponding to putative p53-binding site in caspase-1 promoter is specific and dependent on p53.

3.2.9 Regulation of caspase-1 gene expression by endogenous p53 protein
To address the role of endogenous p53 in regulating endogenous caspase-1 gene expression, MCF-7 cells were treated with doxorubicin, which increased the level of p53 protein (figure not shown). Treatment of MCF-7 cells with doxorubicin enhanced caspase-1 mRNA level 4-5 fold (Fig. 3.8). Similar treatment of MCF-mp53, a clone of MCF-7 cells expressing mutant p53 (His273) or p53 negative HeLa cells did not increase caspase-1 mRNA level (Fig. 3.8). The basal level of caspase-1 mRNA was higher in MCF-mp53 which decreased upon treatment with doxorubicin. Treatment of A549 cells (which have normal p53) with doxorubicin also resulted in an increase in caspase-1 mRNA level (Fig. 3.8). These results showed that endogenous p53 can regulate expression of endogenous caspase-1 gene.

3.2.10 Transactivation of caspase-1 promoter by endogenous p53 protein
In order to determine the role of endogenous p53 in regulating caspase-1 promoter, MCF-7, MCF-mp53 and HeLa cells were transfected with caspase-1 promoter-
### Figure 3.7 Electrophoretic mobility shift assay using the putative p53-binding site sequence of caspase-1 promoter.

**Panel A**

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<th>Oligonucleotide</th>
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<tr>
<td>Caspase-1 Oligo</td>
<td>ataaAGACATGCATATGCATGCACa</td>
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<tr>
<td>Mutant Oligo</td>
<td>ataaAGAAATTATATGAAATTACa</td>
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<tr>
<td>Consensus Oligo</td>
<td>cgagAGACATGCCAGGCATGCCT</td>
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**Panel B**

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Electrophoretic mobility shift assays were done using radiolabeled Casp-1 oligonucleotide with nuclear extracts from MCF-7 cells treated with 500 ng ml⁻¹ of doxorubicin. **Lane 1** is binding without nuclear extract. The arrow shows the p53-specific band that was competed out by a 50-fold excess of unlabeled Casp-1 oligonucleotide (lane 3) and consensus oligonucleotide (lane 4) but not by mutant oligonucleotide (lane 5). The addition of p53 polyclonal antibody (p53 Ab; 1µg) immunodepleted the shifted band (lane 6).
Figure 3.8 Regulation of caspase-1 gene expression by endogenous p53. Indicated cells were treated with 500 ng ml\(^{-1}\) of doxorubicin for 24 and 48h. After RNA isolation caspase-1 mRNA levels were analysed by semiquantitative RT-PCR. \textit{U} indicates untreated cells.
reporter plasmid and after 24 hours treated with doxorubicin for 40 or 48 hours. Doxorubicin treatment resulted in 4-5-fold increase in caspase-1 promoter activity in MCF-7 cells but not in MCF-mp53 or HeLa cells (Fig. 3.9). These results showed that endogenous wild-type p53 can also activate transcription from caspase-1 promoter which is inhibited by His273 mutant of p53.

3.2.11 PTP-S2-mediated p53 upregulation causes transactivation of caspase-1 promoter
To further support our previous results, 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. Reporter plasmids containing promoter region of human caspase-1 (-182 to +42) upstream of CAT cDNA having wild-type p53-binding sites (pCAT-ICE-WT) and mutated p53-binding sites (pCAT-ICE-MT) were used. As shown in Fig 3.10A, PTP-S2 activated the reporter-promoter having wild-type p53-binding sites (upto 3-fold) but not the reporter construct where p53-binding sites were abolished. Next we tested whether PTP-S2 was able to transactivate pCAT-ICE-WT in isogenic background where p53-function was compromised. For this purpose, we used a clone of MCF-7 cells (MCF-mp53) expressing a dominant-inhibitor of wild-type p53 (His273) in which doxorubicin induced activation of p53-responsive promoter and activation of p53-target gene products is obliterated (Fig 3.8; 3.9). PTP-S2 expression was unable to transactivate pCAT-ICE-WT construct in MCF-mp53 cells (Fig 3.10B). This difference in activation of pCAT-ICE-WT by PTP-S2 was not due to variation in the protein levels of exogenously expressed protein as shown in Fig 3.10C. This data indicates that PTP-S2-induced transcriptional activation of human caspase-1 promoter is through p53-binding sites and is dependent on the functional p53 protein in the cell.

3.2.12 PTP-S2 expression does not increase caspase-1 expression in p53 null cells
To further assess the role of p53 status of cells in induction of caspase-1 by PTP-S2, we used a clone of HeLa cells in which PTP-S2 expression is induced by withdrawal of tetracycline. HeLa cells are functionally null for p53 due to presence of human
Figure 3.9 Transactivation of caspase-1 promoter by up-regulating endogenous p53. The indicated cells were cotransfected with pCAT-ICE-WT and pCMV.SPORT-βGal, and 24h post-transfection they were treated with 500 ng ml⁻¹ of doxorubicin for 40 and 48h. CAT activities relative to untreated control (C) are shown (n=3).
Figure 3.10 Transactivation of caspase-1 promoter by PTP-S2 overexpression. Panel A, effect of disruption of the p53-binding site in caspase-1 promoter on its transactivation by PTP-S2. CAT activities relative to control (pTRE) are shown (n=3). Panel B, pCMV.SPORT-βGal plasmid and pCAT-ICE-WT plasmids were cotransfected, along with PTP-S2 (pTRE-S2) or control (pTRE) plasmids in MCF-mp53 cells. CAT activities relative to control (pTRE) are shown (n=3). Panel C, expression of PTP-S2 in MCF-7 and MCF-mp53 cells. Western blotting was performed on total proteins isolated from MCF-7 and MCF-mp53 cells, induced (ind) and uninduced (unind) for PTP-S2 expression using antibodies against PTP-S2, and Cdk-2.
papilloma virus E6 oncoprotein which, binds to p53 and targets it to ubiquitin-dependent degradation. Induction of PTP-S2 protein and mRNA upon removal of tetracycline is shown (Fig 3.11A, B). Surprisingly expression of PTP-S2 in HeLa cells leads to down-regulation of caspase-1 mRNA as determined by RT-PCR (Fig 3.11C) as well as down-regulation of human caspase-1 promoter-reporter activity (Fig 3.11D). These results indicate that presence of functional p53 is required for PTP-S2 to increase the caspase-1 gene expression and PTP-S2 can affect transcription of caspase-1 through p53-independent mechanism.

3.2.13 Abrogation of p53 function attenuates PTP-S2-induced apoptosis

Ectopic expression of caspase-1 has been shown to induce apoptosis (Miura et al., 1993; Alnemri et al., 1995; Mao et al., 1998). As shown in Fig 3.12 A. overexpression of caspase-1 induced apoptosis in MCF-7 cells. We determined the extent of apoptosis induced by PTP-S2 overexpression in MCF-7, MCF-mp53, and HeLa cells using enzyme reporter-based apoptosis assay as described by Miura and Yuan (2000). In this assay, a reporter plasmid such as β-Gal is cotransfected with the gene of interest (in this case PTP-S2) and the decrease in β-Gal activity provides a measure of apoptosis. The PTP-S2 overexpression induced apoptosis in MCF-7 cells and not in MCF-mp53 and HeLa cells (Fig 3.12B). These results confirm earlier results which, showed that the presence of functional p53 is necessary for PTP-S2-induced apoptosis (Radha et al., 1999). In addition, the induction of apoptosis by PTP-S2 correlates with the increase in caspase-1 gene expression in these cell lines.

3.3 DISCUSSION

The results presented in this chapter showed that overexpression of PTP-S2 increased caspase-1 mRNA level several fold in MCF-7 cells. This increase in mRNA level was due to the transactivation of caspase-1 promoter. The transactivation of caspase-1 promoter by PTP-S2 was abolished in the cells where p53 function was compromised either by a mutant (His273) p53 in MCF-mp53 cells or by human papillomavirus E6 oncoprotein in HeLa cells. The increase in promoter activity by PTP-S2 was abrogated by disruption of p53-binding sites in the promoter. Thus, p53 signaling appears to be required for upregulation of caspase-1 gene
Figure 3.11 Effect of PTP-S2 on caspase-1 gene expression in HeLa cells.  
Panel A and B, induction of PTP-S2 expression in double-stable PTP-S2 clone (C14) upon removal of tetracycline. A, cells were induced for PTP-S2 expression for 72 h, fixed and stained using anti-PTP-S2 antibody. B, Northern blot analysis of total RNA isolated from uninduced (unind) and induced (ind) cells using specific probes for PTP-S2 and GAPDH. Panel C, After total RNA isolation caspase-1 and Bax mRNA levels were analysed by semiquantitative RT-PCR. Panel D, pCMV.SPORT-βGal plasmid and pCAT-ICE-WT plasmids were cotransfected, along with PTP-S2 (pTRE-S2) or control (pTRE) plasmids in HeLa cells. CAT activities relative to control (pTRE) are shown (n = 2).
Figure 3.12 **Effect of functional p53 on PTP-S2 induced apoptosis.** Panel A, caspase-1 overexpression induces apoptosis in MCF-7 cells. pCMV.SPORT-βGal plasmid and Caspase-1 plasmids were cotransfected in MCF-7 cells and 48h post-transfection cells were harvested and β-Gal assay was performed (n=3). Panel B, PTP-S2-induced apoptosis in the indicated cells as determined by β-Gal based reporter apoptosis assay (n=3).
expression by PTP-S2. In addition, overexpression of PTP-S2 increased p53 protein level in MCF-7 cells. The increase in p53 protein was through post-transcriptional mechanisms as there was no change in mRNA level of p53. The increased p53 protein showed nuclear localization. The PTP-S2-induced activation of p53 protein does not require p14ARF since MCF-7 cells lack the p14ARF function due to a deletion in the gene (Stott et al., 1998). These results suggest that PTP-S2 can engage p53 via p14ARF-independent mechanisms. Collectively, our results identify a novel afferent signaling pathway to p53 that appears to operate independent of p14ARF.

Specific phosphorylation, dephosphorylation and acetylation events have been reported to activate p53. Phosphorylation of the N-terminus of p53, which has been shown to occur after DNA damage, appears to reduce its affinity for binding to MDM2, which may lead to stabilization of p53 (Shieh et al., 1997). DNA damage causes phosphorylation of serine residues in the amino terminus of p53. In particular, serine-15 has been shown to be phosphorylated in response to DNA damage by ionizing irradiation (IR) or ultraviolet irradiation (UV). Recent experiments have revealed that ATM may associate with p53 and phosphorylate its amino-terminus directly (Khanna et al., 1998). Other kinases which can phosphorylate p53 include cyclin-dependent kinase (CDKs), casein kinase I (CK I), casein kinase II (CK II), protein kinase C (PKC), mitogen activated protein kinase, Jun amino-terminal kinase (JNK), Raf kinase (reviewed in Meek, 1994).

Serine/threonine protein phosphatase type 5 (PP5) has been shown to modulate the phosphorylation and DNA-binding activity of p53 (Zuo et al., 1998). ATM also appears to be required for IR-induced dephosphorylation of p53 Serine-376 which allows specific binding of 14-3-3 proteins to p53 and leads to an increase in sequence-specific DNA-binding activity of p53 (Waterman et al., 1998). Recently, acetylation of the C-terminus of p53 by CREB-binding protein (p300/CBP) was shown to enhance sequence-specific DNA-binding by p53 (Gu et al., 1997). p300/CBP are closely related histone acetyl transferases (HATs) (Bannister et al., 1996; Ogryzko et al., 1996) that interact with p53 and function as coactivators for p53-mediated transcription (Avantaggiati et al., 1997; Gu et al., 1997; Lill et al., 1997).
Figure 3.13 Proposed model for the mechanism of activation of p53 during PTP-S2-induced apoptosis.
1997). The activation of sequence-specific DNA-binding by p53 following DNA damage may involve sequential N-terminal phosphorylation followed by C-terminal acetylation by the coactivator p300 following DNA damage (Sakaguchi et al., 1998). With the use of phosphorylated or acetylated peptide specific antibodies, it has been shown that p300 acetylates Lys-382 while the p300/CBP-associated factor (PCAF) acetylates Lys-320 of p53 and that either acetylation leads to enhanced sequence-specific DNA-binding in vitro (Sakaguchi et al., 1998). p53 was found to be acetylated at Lys-382 and phosphorylated at Ser-33 and Ser-37 in vivo after exposure of cells to UV light or ionizing radiation (Sakaguchi et al., 1998).

Several viral and cellular oncogenes have been shown to stabilize p53. The mechanism of p53 stabilization in response to viral oncogene expression has not been clearly understood until recently when p14ARF (p19ARF in mice) a product of human INK4a/ARF locus translated in an alternate reading frame (Kamijo et al., 1997; Stone et al., 1995) was identified. It was found that the ability of E1A to induce p53 is severely compromised in p19ARF-null cells (Stanchina et al., 1998). p14ARF is a tumor suppressor, which can induce cell cycle arrest in a p53-dependent manner. p14ARF is localized to nucleolus and can physically associate with MDM2 to alter the p53 levels and activity (Pomerantz et al., 1998). p14ARF relocates and sequesters MDM2 in nucleolus, thereby inhibiting MDM2-mediated ubiquitination of p53; as a consequence level of p53 in nucleoplasam rise (Weber et al., 1999; Tao and Levine, 1999). Certain cellular oncogenes like c-myc and ras have also been shown to stabilize p53 in a p14ARF-dependent manner (Zindy et al., 1998; Palmero et al., 1998). Recently, Raf has been shown to activate p53 independent of p19ARF in murine keratinocytes (Roper et al., 2001).

3.3.1 Possible mechanism of stabilization and activation of p53 by PTP-S2

p53 is a short-lived protein with a half-life of about 20 minutes and is often undetectable in normal cells. It is believed that ubiquitin-mediated proteolysis plays a role in the rapid turnover of p53 protein (Chowdary et al., 1994). The amount of p53 in cells is determined mainly by the rate at which it is degraded, rather than the rate at which it is made (Vogelstein et al., 2001). MDM2 is a transcriptional target of p53 and mediates ubiquitin-dependent degradation of p53 (Perry et al., 1993). Thus,
transcriptional activation of MDM2 by p53 produces a potent negative-feedback regulatory loop. The importance of negative regulation of p53 by MDM2 is highlighted by the fact that homozygous deletion of MDM2 in mice results in embryonic lethality, which is rescued by simultaneous homozygous deletion of p53 (Luna et al., 1995; Jones et al., 1995). The phosphatidylinositol 3-kinase/Akt pathway has been shown to promote translocation of MDM2 from cytoplasm to nucleus (Mayo and Donner, 2001). MDM2 is phosphorylated on serine 166 and 186 by Akt and this phosphorylation is required for nuclear entry of MDM2 (Mayo and Donner, 2001). This is a critical step in the pathway through which MDM2 inhibits the transcriptional activity of p53 and targets it for degradation. Further, constitutively active PI3K and PKB/Akt are capable of significantly delaying the onset of p53-mediated, transcriptionally dependent apoptosis (Sabbatini and McCormick, 1999).

Using substrate-trap mutants EGFR and Shc were identified as the substrates of TC-PTP in EGF-treated Cos-1 cells (Tiganis et al., 1998; 1999). Recently, TC45, a human homologue of PTP-S2 has been shown to recognize ΔEGFR, a constitutively active receptor as a cellular substrate and inhibit mitogen-activated protein kinase ERK2 and phosphatidylinositol 3-kinase signaling (Klingler-Hoffman et al., 2001). Thus, we propose that PTP-S2 may increase the p53 protein by dephosphorylating the EGFR and inhibiting the activation of PI3-kinase/Akt/MDM2 pathway (Fig 3.13). PTP-S2-induced increase in p53 function may occur via the prevention of MDM2 entry into the nucleus, and thereby reducing the ubiquitin-mediated degradation of p53. This hypothesis predicts that overexpression of PTP-S2 would result in inhibition of phosphorylation of MDM2 thereby resulting in cytoplasmic localization of MDM2. This prediction can be easily tested by immunostaining of PTP-S2 expressing cells with anti-MDM2 antibody.

3.3.2 Caspase-1 as transcriptional target of p53
Several p53-responsive genes have been identified by using different approaches and various cell types (Polyak et al., 1997; Jian et al., 1999; Zaho et al., 2000; Kannan et al., 2001). These p53-responsive genes include various functional categories such as those involved in apoptosis, cell cycle, signal transduction, angiogenesis, etc. (Zhao et al., 2000). However none of the members of the caspase
Figure 3.14 Schematic representation of results presented and their possible biological implications.
family have been identified as a transcriptional target of p53. Our results showed that human caspase-1 is direct transcriptional target of tumor suppressor p53. *This is the first report describing any caspase family member as a transcriptional target of tumor suppressor p53* (Gupta et al., 2001). The induction of various target genes by p53 is dependent on the type of inducer and even with same inducer it is cell-type specific (Zhao et al., 2000). The PTP-S2-induced p53 protein increased the mRNA level of p21 and caspase-1 without affecting the level of Bax mRNA. The activation of caspase-1 transcription by PTP-S2 overexpression correlated with the induction of apoptosis by PTP-S2.

Ectopic expression of caspase-1 induced apoptosis in MCF-7 cells. The wild-type p53-induced apoptosis in MCF-7 cells was partially inhibited (50% inhibition) by YVAD-cmk (which preferentially inhibits caspase-1) but not by caspase-3 family inhibitor DEVD-cmk. Doxorubicin-induced apoptosis in MCF-7 cells was also partially inhibited (45% inhibition) by YVAD-cmk and not by DEVD-cmk (Gupta et al., 2001). These observations suggest that caspase-1 contributes in part to p53-mediated apoptosis. Apoptotic pathways are cell type and stimulus specific and it is likely that caspase-1, along with other transcriptional targets may play a role in PTP-S2-mediated apoptosis in MCF-7 cells.

The primary role of caspase-1 is in the production of proinflammatory cytokines interleukin-1β, interleukin-16 and interleukin-18 (Los et al., 1999). Wild-type p53 is overexpressed in several inflammatory diseases including rheumatoid arthritis, Ulcerative colitis, chronic pancreatitis, idiopathic pulmonary fibrosis, lymphocytic thyroiditis, *Helicobacter pylori*- associated gastritis (reviewed in Tak et al., 2000) but its potential role in inflammation is not understood. *Our results, showing that caspase-1 is transcriptionally activated by p53, suggest that p53 has a role in inflammation. Mutational inactivation of p53 in human tumors would, therefore, lead to reduced inflammatory response, in addition to resistance to apoptosis.*