p53 facilitates degradation of human T-cell leukaemia virus type I Tax-binding protein through a proteasome-dependent pathway

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Human T-cell leukaemia virus type 1 (HTLV-I), the aetiological agent of adult T-cell leukaemia (ATL) and tropical spastic paraparesis (TSP/HAM), transforms human T-cells in vivo and in vitro. The Tax protein of HTLV-I is essential for cellular transformation as well as viral and cellular gene transactivation. The interaction of Tax with cellular proteins is critical for these functions. We previously isolated and characterized a novel Tax-binding protein, TRX (TAX1BP2), by screening a Jurkat T-cell cDNA library. In the present study, we present evidence that the tumour suppressor p53 targets the TRX protein for proteasome degradation. Pulse-chase experiments revealed that p53 enhanced the degradation of TRX protein and reduced the half-life from 2-0 to 0-25 h. p53 mutants R248W and R273H enhance TRX degradation suggesting a transcriptionally independent mechanism. Both HTLV-I Tax and the proteasome-specific inhibitor MG132 inhibited p53-mediated TRX protein degradation. These results suggest that TRX degradation is mediated through activation of the proteasome protein degradation pathway independent of transcriptional function of p53. Our results provide the first experimental evidence that Tax inhibits transcription-dependent and independent functions of p53.

INTRODUCTION

Human T-cell leukaemia virus type 1 (HTLV-I) is the aetiological agent of adult T-cell leukaemia (ATL) and the neurological disease tropical spastic paraparesis (TSP/HAM) (Gessain et al., 1985; Osame et al., 1986; Poiesz et al., 1980; Yoshida et al., 1984). HTLV-I provirus encodes a 40 kDa oncoprotein, Tax, which plays a key role in virus replication and cellular transformation. Tax activates transcription of viral as well as cellular genes, including IL-2, IL-2Rz, IL-1, IL-6, IL-8, IL-10, IL-15, GMCSF, c-fos and c-jun, and negatively regulates β-polimerase, a DNA-repair enzyme and c-myb (Azimi et al., 1998; Brady, 1996; Fujii et al., 1991; Jeang et al., 1990; Mori et al., 1998; Mori & Prager, 1998; Nicot et al., 2000; Nimer, 1991; Sawada et al., 1992; Siekevitz et al., 1987; Yamashita et al., 1994). The deregulated expression of some of these genes likely plays an important role in transformation.

Mutation and inactivation of p53 are common in human cancers, occurring in over half of all human tumours (Harris, 1993; Hollstein et al., 1991; Ko & Prives, 1996; Levine, 1997; Nigro et al., 1989; Prives & Manley, 2001; Ryan et al., 2001). In response to various types of DNA damage and cell stress signals, the p53 tumour suppressor functions to integrate cellular responses including growth arrest at the G1 phase of the cell cycle or apoptosis (Amundson et al., 1998; Bates & Vousden, 1999; el-Deiry et al., 1993; Gottlieb & Oren, 1998b; Harper et al., 1993; Polyak et al., 1997; Selivanova & Wiman, 1995; Shen & White, 2001; Wu et al., 1999). The biochemical activity required for p53 tumour suppression and the responses to DNA damage involve the ability of p53 to bind DNA in a sequence-specific manner and function as a transcriptional activator (el-Deiry et al., 1992; Fields & Jang, 1990; Liu & Kulesz-Martin, 2001; Raycroft et al., 1990). Expression of p53 in cells activates, through consensus p53 binding sites, a number of genes involved in p53-induced cell cycle arrest or apoptosis, including GADD45, WAF1, MDM2, Bax and PIG3 (Flatt et al., 2000; Levine, 1997; Yu et al., 1999). The p53 protein is a key regulator of apoptosis under a variety of physiological and pathological conditions (Bates & Vousden, 1999; Kastan et al., 1995; Polyak et al., 1997).

In view of recent results, it appears that p53 may also regulate cell cycle progression through targeted degradation of cell cycle regulatory proteins. Gottlieb & Oren (1998a) have reported that p53 facilitates pRb cleavage and degradation by activating the caspase protease pathway. Wild-type p53 has also been shown to accelerate degradation of
cellular proteins including FLIP, which inhibits apoptosis (Fukazawa et al., 2001); β-catenin, which is implicated in tumour development (Sadot et al., 2001); delta-Np63, which is linked to accelerated tumorigenesis (Ratovitski et al., 2001); and BRCAl, the breast and ovarian cancer susceptibility gene, which has been suggested to be involved in gene transcription and DNA repair (Irminger-Finger et al., 1999; Choi, 2001). In addition, Ravi et al. (2000) reported that p53 enhances degradation of the hypoxia-inducible factor 1α, which is involved in tumour angiogenesis.

Tax-binding proteins, including CREB, p16, CBP/p300, NF-κB, cyclin D and Mad1, which play pivotal roles in Tax transactivation and cell cycle regulation, have been identified (Adya et al., 1994; Bex et al., 1998; Brady, 1996; Kanno et al., 1994; Kashanchi et al., 1998; Kwok et al., 1996; Neuveut et al., 1998; Suzuki et al., 1996; Van Orden et al., 1999). We previously reported the isolation of a Tax-binding protein, TRX (TRAXBP2), following screening of a Jurkat T-cell cDNA expression library. Direct interaction between Tax and TRX was demonstrated using Western blot and coimmunoprecipitation assays (Mireskandari et al., 1996). We further demonstrated that TRX RNA was expressed ubiquitously in a number of cell lines and human tissues. In the present report, we demonstrate that the tumour suppressor p53 inhibits TRX expression by targeting and enhancing TRX degradation through a ubiquitin proteasome-mediated pathway. Similar to its ability to inhibit p53 transactivation, Tax inhibits the transcription-independent p53-mediated TRX proteolysis.

METHODS

**Cells.** Cos-1, HeLa and p53-negative human osteosarcoma cells (Saos-2) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM l-glutamine and antibiotics (100 U penicillin ml⁻¹ and 100 U streptomycin ml⁻¹). The p53-negative human T-cell line Jurkat was cultured in RPMI supplemented with 10% FBS, 2 mM glutamine and 100 U penicillin ml⁻¹ and 100 U streptomycin ml⁻¹.

**Plasmids.** The TRX cDNA (Mireskandari et al., 1996) was cloned into the PCRII vector (Invitrogen) and was amplified using Pfu DNA polymerase. The forward primer contained the 5'coding sequences of TRX, with an additional ATG codon and Kpnl site. The reverse primer contained 3'coding sequences of TRX cDNA and a Xhol site. The PCR product was gel-purified and digested with KpnI and XhoI. The eukaryotic expression vector pcDNA3.1 His A or B (Invitrogen), containing the human cytomegalovirus (CMV) promoter and tag sequence for antibody detection, was digested with KpnI and XhoI and the TRX cDNA fragment inserted in-frame. TRX protein expression was tested by in vitro transcription–translation using a rabbit reticulocyte lysate system (Promega), immunoprecipitation with a tag antibody (anti-Xpress, Invitrogen), or by Western blotting with anti-TRX antibody (rabbit polyclonal). TRX protein expression was also confirmed by transfecting Jurkat, Cos-1, HeLa and Saos-2 cells, followed by Western blotting and immunostaining with anti-tag or anti-TRX antibody. Tax expression vectors pc-Tax (wild-type) and Tax mutant M47 (L139R-L320S) were kindly provided by W. C. Greene (Smith & Greene, 1990). The expression vector for p53 mutants p53R248W and p53R273H, and the p53 responsive element-containing reporter plasmids pG13-Luc and MDM2-Luc, were gifts from V. Vogelstein (Johns Hopkins University, Maryland, MD), and pCMVp53, which expresses wild-type (wt) human p53 from the HCMV immediate-early promoter, was a gift from S. J. Kim (National Cancer Institute, Bethesda, MD).

**Transfection.** Transient transfection experiments with Cos-1, HeLa, Saos-2 and Jurkat cells were performed using either electroporation (Ausubel et al., 1989) or FuGene methods as described in the manufacturer’s instructions (Roche Diagnostics). Briefly, cells were washed with RPMI without FBS and resuspended in 300 μl of the same media at a concentration of 5 × 10⁶ per sample. The cells were electroporated (Bio-Rad Gene pulser) using 230 to 250 V at a capacitance of 975 μF. The amount of DNA transfected was normalized by the addition of vector control plasmid. TRX, Tax, p53, and p53 mutants R248W and R273H protein expression was assayed by Western blot as described below.

**Western blotting.** Cells were washed with cold PBS, treated with PBS containing 2 mM EDTA, scraped and lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, containing 1 mM PMSE, aprotinin (1 μg ml⁻¹), leupeptin (1 μg ml⁻¹) and 5 mM sodium fluoride at 4°C for 1 h. The lysate was cleared by centrifugation in a microcentrifuge at 14000 r.p.m. for 15 min and the protein concentration was determined accordingly (Bio-Rad). Cell lysates (15 to 30 μg) were separated on 12% or 4 to 20% SDS–polyacrylamide gradient gels, transferred to an Immobilon membrane (Millipore) and assayed for TRX expression using anti-Xpress Tag antibody (Invitrogen). Tax, p53, mutant p53 (R248W and R273H) and the CMV immediate early 2 86 kDa gene product (IE2) were detected using anti-Tax (Tab 172), anti-p53 (Do-1, pab421) and anti-IE-2 (rabbit polyclonal) antibodies, respectively. Antigen–antibody complexes were detected using the enhanced chemiluminescence system (Amersham).

**RNA isolation and Northern blotting.** RNA was isolated from transfected cells using TRIzol (Gibco-BRL). Fifteen μg of total RNA was used for each Northern blot analysis. RNA was vacuum-dried for 10 min, resuspended in denaturation buffer (10 mM phosphate buffer pH 7.0, 1 × MOPS buffer, 50% formamide, 2-1 M formaldehyde) and incubated at 65°C for 5 min followed by quenching on ice. RNA loading buffer (2 μl) was added to each sample. Samples were run on a 1% agarose gel containing 1 × MOPS and 0.66 M formaldehyde and electrophoresis was performed at 50 V in 1 × MOPS buffer. The RNA was blotted onto a nylon membrane (Micron Separation) using capillary action, and then fixed onto the membrane using a UV-Stratalinker 2400 (Stratagene). A 5'end labelled TRX cDNA probe was used to detect the amount of TRX RNA in each sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech) was used as a control for RNA loading.

**Immunoprecipitation.** Cos-1 cells were co-transfected with TRX alone or in the presence of p53. Cells were pulsed with [35S]methionine and [35S]cysteine (200 μCi ml⁻¹ or 500 μCi per 6 cm plate) for 30 min after 24 h of transfection, washed and chased for 3 h in complete medium. Samples were collected at 0, 0.5, 1-0, 2-0 and 3-0 h post-pulse. Cells were lysed in RIPA buffer, the protein concentration was determined and 500 μg of protein used for immunoprecipitation with an anti-Xpress (anti-Tag) antibody for the detection of TRX. The amount of labelled protein was quantified using the ImageQuant program (Molecular Dynamics).

** Luciferase assay.** To assay for luciferase activity, Saos-2 cells were harvested 24 h after transfection by pG13-Luc or MDM2-Luc with wt p53 or mutants p53R248W or p53R273H. The cells were washed twice in cold PBS, and then lysed in 400 μl of Promega passive lysis buffer. p53R248W and p53R273H. Cells were then gently vortexed and kept on ice for 30 min. The supernatant was clarified by centrifugation at
14 000 r.p.m. for 5 min at 4 °C. The extract was kept at −80 °C until further use. The protein concentration was determined by the Bradford protein assay (Bio-Rad). Equal amounts of protein (5 to 20 μg) were added to 100 μl of luciferase substrate buffer according to the manufacturer’s protocol (Promega); these samples were then analysed in a Berthold LB9500C Luminometer. Luminescence was measured in relative light units (RLU). Luciferase activity was expressed as average light units produced per μg of protein present in the specific cell lysate for each experiment. Each sample was assayed in triplicate and the average value was determined ± standard deviation.

RESULTS

Inhibition of TRX expression by p53

We have previously reported that HTLV-I Tax inhibits the transactivation function of p53 (Pise-Masison et al., 1998, 2000, 2001). In addition we have demonstrated that Tax interacts with a novel cellular protein, TRX (Mireskandari et al., 1996). In a series of experiments designed to test the biological function of TRX on Tax inhibition of p53 function, we observed a significant effect of p53 on TRX expression. First, cells were transfected with the TRX/pcDNA3.1 expression plasmid and TRX protein was analysed 24 h post-transfection by Western blot using anti-Xpress (Tag) antibody (Fig. 1). While the antibody failed to react with proteins in the control extract (lane 1), a protein migrating at 25 kDa was detected in cells transfected with the TRX expression plasmid (lanes 2 to 4). Increasing amounts of the TRX protein was observed as the amount of TRX plasmid was increased in the transfection mix. The identity of the protein was confirmed by Western blot analysis with a peptide antibody raised against TRX protein (data not shown).

Subsequently, TRX was transfected into Cos-1 cells in the presence or absence of Tax and p53. Cotransfection of Tax with TRX did not significantly alter the level of TRX protein expression (Fig. 2A; compare lanes 2 and 7). Interestingly, we observed that cotransfection of p53 decreased the level of TRX protein in Cos-1 cells (Fig. 2A, lanes 2 and 8). The ability of p53 to inhibit TRX expression was dose-dependent (data not shown).

We next tested whether p53 inhibited TRX expression in other cell lines including the p53-negative osteosarcoma cell line, Saos-2. Consistent with the results presented above, these experiments showed that p53 inhibited TRX protein expression. Complete inhibition of TRX expression was observed at 1 μg plasmid DNA in the transfected cells (Fig. 2B, lane 5). The extracts were also used for Western blot analysis with a p53-specific antibody. The results of this experiment demonstrate that the level of p53 expression is slightly increased in samples containing TRX (Fig. 2C; compare lanes 2 and 3 with 5 and 6).

Fig. 1. Expression of TRX protein in Cos-1 cells. Cos-1 cells were transfected by electroporation as described with TRX/pcDNA3.1 expression plasmid and cells were harvested 24 h post-transfection. TRX expression was determined by Western blot and immunostaining using anti-Xpress (Tag) antibody, which detects TRX protein, followed by chemiluminescence detection. pCMV was used to normalize the amount of transfected DNA. These figures represent one of five separate experiments.

Fig. 2. Inhibition of the steady state level of TRX protein by the tumour suppressor protein p53. (A) Cos-1 cells were transfected with TRX expression plasmid alone or in the presence of either p53 or Tax expression plasmids. TRX expression was determined by Western blot followed by immunostaining. (B and C) Dose-dependent inhibition of TRX expression by p53 in Saos-2 cells (p53 null). Cells were transfected with TRX expression plasmid (10 μg per sample) in the presence of p53 expression plasmid at 1-0 or 0-3 μg per sample. Cells were harvested 24 h post-transfection and TRX (B) and p53 (C) expression was determined by Western blot analysis. pCMV was used to normalize the amount of transfected DNA. These figures represent one of five separate experiments.
Effect of p53 on TRX RNA expression

It was of interest to determine if the p53 effect on TRX expression was at the transcriptional or post-transcriptional level. Cos-1 cells were cotransfected with TRX/pcDNA3.1 in the presence or absence of p53. Total RNA was prepared and assayed for the level of TRX RNA with a TRX-specific probe. TRX mRNA was detected in cells transfected with TRX plasmid, but not the control cells (Fig. 3A, lanes 1 to 3). The level of TRX mRNA expression was similar in the presence and absence of p53 (lanes 2 and 4), indicating that p53 did not have any significant effect on TRX RNA expression. A control hybridization with the GAPDH probe demonstrated that equal amounts of RNA were present in all of the samples. The slight decrease in GAPDH RNA in lane 4 is not significant. These results suggest that the p53 effect is at the post-transcriptional level. Due to the high level of TRX mRNA produced from the replicating plasmid transfected into the cells, the endogenous 2-3 kb TRX mRNA is not detected in the exposure shown in Fig. 3.

p53 decreases the half-life of TRX protein

To determine if p53 affected the turnover or half-life of TRX protein in the cell, a pulse–chase experiment was performed. Cos-1 cells were transfected with TRX or TRX plus p53. At 24 h post-transfection, cells were pulsed for 30 min with 500 μCi ml⁻¹ [³⁵S]methionine and [³⁵S]cysteine and chased for 0 to 3 h. Cell extracts were immunoprecipitated with an anti-Xpress Tag antibody. The labelled

Fig. 3. p53 does not inhibit TRX RNA expression. Cos-1 cells were transfected with TRX/pcDNA3.1 (4 μg per sample) alone or in the presence of p53 (2 μg per sample). Cells were harvested at 48 h post-transfection. Total RNA was isolated as described. (A) 15 μg total RNA from each sample was resolved in a 1% agarose/formaldehyde gel, transferred onto nylon membrane and hybridized with a TRX cDNA probe labelled with [³²P]dCTP. (B) The same blot was reprobed with a cDNA probe for the internal housekeeping gene GAPDH.

Fig. 4. Comparison of TRX protein half-life in the presence and absence of p53 by pulse–chase analysis. The half-life of TRX protein was determined by pulse–chase experiments. Cells transfected with either TRX alone or in the presence of p53 were metabolically labelled with [³⁵S]methionine and [³⁵S]cysteine. The cells were pulsed for 30 min with 500 μCi per plate in 25 ml medium, washed and chased for 0 to 3 h. 500 μg total protein (cell lysate) from each sample was used for immunoprecipitation by anti-Xpress (Tag) antibody which detects transfected TRX. Proteins were separated by electrophoresis in 12% SDS-PAGE gels. Gels were analysed using the Molecular Dynamics PhosphorImager and ImageQuant program. These figures represent one of two separate experiments.
immunoprecipitates were then analysed by SDS-PAGE and the radioactivity was quantified using the PhosphorImager ImageQuant program. The results demonstrated that equal amounts of TRX protein were present in the TRX- or TRX plus p53-transfected cells at the 0 h time point (Fig. 4A, lanes 1 and 6), indicating that p53 did not affect the translation of TRX mRNA. Remarkably, the TRX protein was much less stable in the p53-expressing cells. In the absence of p53, TRX protein was degraded gradually with a half-life of approximately 2.0 h (Fig. 4A, lanes 1 to 5; Fig. 4B). In the presence of p53, the half-life of TRX was 0.25 h (Fig. 4A, lanes 6 to 10; Fig. 4B). These results suggest that p53 decreases the steady state level of TRX protein by enhancing degradation of the protein. In these studies, we noted that the anti-Tag antibody immunoprecipitated several lower molecular mass proteins. The fact that these bands did not react with the anti-Tag antibody in Western blot analysis (Fig. 1) suggests that the proteins may be proteolytic products or TRX-associated proteins that coimmunoprecipitate with TRX.

**Transcription-independent function of p53 targets TRX degradation**

Next, we examined the activity of transcriptionally inactive p53 point mutants R248W and R273H on TRX degradation in Cos-1 cells. The results indicated that similar to wt p53 the p53 mutants also increased TRX degradation (Fig. 5A). The transcriptional activity of wt p53 and mutants R248W and R273H were confirmed by cotransfecting the p53 plasmids with pG13-Luc (which contains 13 x p53-responsive elements) or MDM2-Luc into p53

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![Fig. 5. Transactivation defective p53 mutants retain the ability to target TRX degradation.](http://vir.sgmjournals.org)

(A) Cos-1 cells were transfected with TRX expression plasmid alone or in the presence of either wt p53 or transcriptionally inactive mutants p53R248W or p53R273H. TRX expression was determined by Western blot followed by immunostaining as described (B) and (C) Saos-2 cells were cotransfected with p53 responsive element-containing reporter plasmids pG13-Luc (B) or MDM2-Luc (C) in absence or presence of wt p53 or mutant p53R248W or p53R273H. The relative luciferase activity was determined in triplicate ± standard deviation. The control luciferase activity for pG13-Luc and MDM2-Luc was 1818 and 8708 units, respectively. (D) 35 S-labelled TRX was synthesized in rabbit reticulocyte lysate and incubated with purified GST or GST–p53. Following incubation, bound proteins were analysed by SDS-PAGE and autoradiography. The TRX protein band is indicated.

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**Fig. 5.** Transactivation defective p53 mutants retain the ability to target TRX degradation. (A) Cos-1 cells were transfected with TRX expression plasmid alone or in the presence of either wt p53 or transcriptionally inactive mutants p53R248W or p53R273H. TRX expression was determined by Western blot followed by immunostaining as described (B) and (C) Saos-2 cells were cotransfected with p53 responsive element-containing reporter plasmids pG13-Luc (B) or MDM2-Luc (C) in absence or presence of wt p53 or mutant p53R248W or p53R273H. The relative luciferase activity was determined in triplicate ± standard deviation. The control luciferase activity for pG13-Luc and MDM2-Luc was 1818 and 8708 units, respectively. (D) 35 S-labelled TRX was synthesized in rabbit reticulocyte lysate and incubated with purified GST or GST–p53. Following incubation, bound proteins were analysed by SDS-PAGE and autoradiography. The TRX protein band is indicated.
negative Saos-2 cells (Fig. 5B and C). The results showed that wt p53 activated p53RE-containing pG13-Luc (153-fold) (Fig. 5B) and MDM2-Luc (25-fold) (Fig. 5C). As expected, p53 mutants R248W and p53R273H failed to activate the p53RE-containing reporter plasmid pG13-Luc or MDM2-Luc. These results suggest that p53-induced TRX protein degradation is mediated through a p53 transcription-independent pathway.

To analyse the potential interaction between TRX and p53, we performed in vitro protein binding assays. 35S-labelled TRX was prepared using the rabbit reticulocyte lysate system. TRX was incubated with purified GST or GST–p53 protein. After centrifugation and washing of the pellets to remove unbound protein, SDS-gel analysis of the proteins demonstrated that TRX bound to GST–p53 but not the GST control (Fig. 5D).

TRX degradation is mediated through the proteasome pathway

Next, we wanted to determine whether p53-mediated TRX degradation was regulated through a specific proteolytic pathway. The proteasome-specific inhibitor MG132 and the cysteine protease inhibitor E64 were added to transfected cells at concentrations of 3 and 10 μM for 16 h. Consistent with previous reports, these conditions are not toxic to cells as shown when trypan blue exclusion is used to measure cell viability (Magae et al., 1997; Meriin et al., 1998) (data not shown). Interestingly, the results show that the proteasome-specific inhibitor MG132 could block p53-mediated TRX protein degradation (Fig. 6A, compare lanes 1, 3 and 7). Complete protection of TRX degradation was achieved at an MG132 concentration of 10 μM. In contrast, the cysteine protease inhibitor E64 (lanes 4 and 5) failed to inhibit TRX degradation. In parallel experiments, the calpain-specific inhibitor ALLN also failed to prevent p53-mediated TRX degradation (data not shown). These results suggest that p53 targets TRX proteolysis through the proteasome degradation pathway.

A Western blot analysis of the extracts with a p53-specific antibody is presented in Fig. 6(B). Consistent with the results presented in Fig. 2(C), coexpression of TRX with p53 leads to a slight increase in the steady state level of p53 in the cells (lanes 2 and 3). No further increase in the level of p53 expression was observed when E64 or MG132 was added to the cells.

Specificity of TRX protein degradation by p53

To determine if p53-mediated degradation of TRX was specific, we used the CMV IE-2 86 kDa gene product. IE-2 transactivates the CMV immediate early gene and has been shown to deregulate cell cycle and apoptosis pathways (Castillo et al., 2000; Salvant et al., 1998; Wiebusch & Hagemeier, 1999; Zhu et al., 1995). The IE-2 expression vector was transfected into Saos-2 cells in the presence or absence of p53. As an experimental control, cells were transfected with TRX alone or in the presence of p53. Consistent with results presented above, the expression of TRX protein was reduced in the presence of p53 (Fig. 7B, lanes 2 and 3). In contrast, the level of IE-2 protein expression was similar in the presence and absence of p53 (Fig. 7A, lanes 4 and 5). Two background protein bands were routinely detected in the Western blots with the IE-2 antibody (Fig. 7A). The level of reactivity, however, did not change significantly in the different experimental conditions and thus does not interfere with the interpretation of results.

Tax inhibits p53-mediated degradation of TRX

We have shown that Tax inhibits the transactivation function of p53 (Pise-Masison et al., 1998, 2000, 2001). To test whether Tax inhibited the p53-mediated degradation of TRX, transfection experiments were repeated in the presence and absence of a Tax expression vector. Similar to the results presented above, we observed that the level

Fig. 6. TRX degradation is through the proteasome pathway. Saos-2 (p53 null) cells were transfected with TRX alone or cotransfected with p53 and cultured for 24 h. Transfected cells were treated with medium alone, the cysteine protease inhibitor E64, or the 26S proteasome-specific inhibitor MG132, at concentrations of 3 and 10 μM. Cells were harvested and TRX (A) and p53 (B) protein was analysed by Western blotting. These figures represent one of five separate experiments.
of TRX protein expression was diminished in the presence of p53 (Fig. 8A, compare lanes 1, 5 and 6). In contrast, cotransfection with Tax resulted in the inhibition of TRX degradation (lanes 7 and 8). This result suggests that Tax is able to inhibit the p53-targeted degradation of TRX protein. Western blot analysis with control p53 and Tax-specific antibodies is shown in Fig. 8(B) and (C), respectively.

To demonstrate the specificity of Tax inhibition of p53 function, we tested whether a Tax mutant was able to inhibit p53-mediated TRX degradation. As demonstrated above, cotransfection of TRX with p53 led to a significant reduction in the level of TRX expression. Quantitative analysis of the blot indicated there was approximately an 85% reduction in TRX expression in the presence of p53. Consistent with the results presented above, we observed that wild-type Tax inhibited p53-mediated TRX degradation (Fig. 9A, lanes 1, 3 and 4). In contrast, Tax mutant M47, which is defective in CREB activation and binding to CBP and PCAF, was significantly less efficient at inhibiting p53-mediated TRX degradation (lane 5). Quantitative analysis of the Western blot indicated there was a 65 to 70% reduction in TRX expression in the presence of p53 and the Tax mutant M47. Consistent with these results, M47 does not inhibit p53 transactivation function in Saos-2 and HeLa cells (Pise-Masison et al., 2001; Mulloy et al., 1998). The inability of M47 to inhibit p53 function was not due to protein expression levels, since an equal or greater level of M47 protein was observed in the cell extracts (Fig. 9B, lanes 4 and 5).

DISCUSSION

The p53 tumour suppressor protein is critical for controlling cell proliferation, protection against oncogenic transformation and is an important regulator of apoptosis under a variety of physiological and pathological conditions. Transcriptional activation of target genes is one of the key mechanisms by which p53 induces cell cycle arrest and apoptosis (Kastan et al., 1995; Levine, 1997). Many lines of evidence support a role for the activation of the cyclin-dependent kinase inhibitor waf1/Cip1/p21 in p53-induced cell cycle arrest (el-Deiry et al., 1993; Harper et al., 1993). Transcriptional activation of Bax (Miyashita & Reed, 1995) and IGF-BP3 (Buckbinder et al., 1995) play a significant role in the apoptosis response pathway. p53 can also induce cell cycle arrest by repressing transcription from a variety of growth-promoting genes, including those of the c-fos, c-jun, c-myc, Rb, MDR1, IL-6 and PCNA (Chin et al., 1992; Ginsberg et al., 1991; Lechner et al., 1992; Ragimov et al., 1993; Santhanam et al., 1991; Shio et al., 1992; Subler et al., 1992; Yamaguchi et al., 1994).

p53 may also regulate tumour development and cell cycle progression through targeted degradation of cell cycle protein expression was diminished in the presence of p53 (Fig. 8A, compare lanes 1, 5 and 6). In contrast, cotransfection with Tax resulted in the inhibition of TRX degradation (lanes 7 and 8). This result suggests that Tax is able to inhibit the p53-targeted degradation of TRX protein. Western blot analysis with control p53 and Tax-specific antibodies is shown in Fig. 8(B) and (C), respectively.

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p53 may also regulate tumour development and cell cycle progression through targeted degradation of cell cycle

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Fig. 7. p53 fails to target CMV IE-2 protein for degradation. Saos-2 cells (p53 null) were transfected with TRX or CMV IE-2 alone or in the presence of p53. Cells were harvested 24 h post-transfection and expression of IE-2 (A) and TRX (B) was determined by Western blot followed by immunostaining with anti-IE-2 for IE-2 detection and anti-Xpress for TRX. These figures represent one of two independent experiments.

Fig. 8. Tax inhibits p53-mediated TRX degradation. Saos-2 cells were transfected with TRX (8 μg) alone or in the presence of p53 at 0.3 and 1.0 μg plasmid DNA. The Tax expression plasmid pc-Tax (7 μg) was cotransfected with TRX and p53 as indicated. Cells were harvested 24 h post-transfection and 30 μg of protein was analysed by Western blot analysis using anti-Xpress antibody for TRX (A), antibody DO-1 for p53 (B) and Tab-172 for Tax (C). These figures represent one of five independent experiments.
cells did not result in cell cycle arrest at either the G1/S or cycle analysis, overexpression of TRX protein in a variety of dependent degradation of the cellular protein TRX. In cell study, we demonstrate that p53 promotes proteasome-

The figure represents one of two independent experiments.

Fig. 9. Tax mutant M47 is unable to block p53-mediated TRX degradation. Saos-2 cells were cotransfected with TRX alone or with p53. The TRX+p53 samples were cotransfected with either wt Tax, or with Tax mutant M47. Cells were harvested 24 h post-transfection and TRX (A) and Tax (B) protein expression were determined by Western blot followed by immunostaining. The figure represents one of two independent experiments.

To understand the mechanism of TRX degradation by p53, we have analysed p53 mutants which are transcriptionally inactive. The results of these studies demonstrate that TRX degradation is a transcription-independent function of p53, since p53R248W and p53R273H stimulate degradation. Ratovitski et al. (2001) showed similar results in the degradation of delta Np63 protein by p53. Of interest, delta Np63 degradation occurred via the caspase pathway. We used cysteine protease inhibitor E64, the 26S- proteasome inhibitor MG132 and the calpain 1 protease inhibitor ALLN. The cysteine protease inhibitor E64 and calpain 1 protease inhibitor ALLN failed to protect the p53-mediated TRX degradation. In contrast, proteasome-specific inhibitor MG132 blocked p53-mediated TRX degradation. The analysis of TRX protein by the PeptideCutter program revealed that there are no caspase (caspase1–caspase10) cutting sites in the TRX protein. In contrast, chymotrypsin (30 cutting sites) and trypsin (20 cutting sites) protease sites are abundant.

The emerging model of the functional interaction of Tax and p53 interaction supports the ability of Tax to inhibit several functions of p53. We and others have previously shown that Tax inhibits the transactivation function of p53 (Ariumi et al., 2000; Cereseto et al., 1996; Lemasson & Nyborg, 2001; Mulloy et al., 1998; Pise-Masison et al., 1998, 2000, 2001; Suzuki et al., 1999; Van Orden et al., 1999). The data presented in this study demonstrate that Tax also blocks transcription-independent functions of p53. It will be of interest to further identify the functional consequences of TRX degradation, interaction with tumour suppressor p53 and its involvement in HTLV-I-induced leukaemogenesis.

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REFERENCES


