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Thioredoxin-1 phosphorylated at T100 is needed for its anti-apoptotic activity in HepG2 cancer cells

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ABSTRACT

Aims: Thioredoxin-1 (Trx) is an important protein involved in the regulation of apoptosis and in enhancing drug resistance in cancer cells. Threonine100 (T100) of Trx was reported to be phosphorylated; however, the role of this phosphorylation in regulating activity remains unresolved. We explored whether and how the phosphorylation of Trx is involved in drug resistance in cancer cells.
Main methods: The levels of phosphorylated Trx were detected by sandwich ELISA. Cell viability was investigated using Alamar Blue assay, and apoptosis was evaluated with Hoechst33258 staining. Western blotting was used to examine the changes in the expression levels of Trx, NF-κB p65 subunit, ASK-1 and 6His tag. Additionally, we took advantage of phorbol-12-myristate-13-acetate (PMA)to activate protein kinase C (PKC) and staurosporine to inhibit PKC.
Key findings: Trx mutated at T100 causes lower survival rate induced by H_2O_2, and higher apoptosis rate induced by cis-platinum and adriamycin in HepG2 cells. T100 of Trx can be phosphorylated through the PKC-dependent pathway. Furthermore, the resistance of anticancer drugs can be decreased when the phosphorylation of T100 in Trx was blocked by staurosporine. Though the Trx-ASK1 complex is not affected, the phosphorylation contributes to the nuclear location of Trx, and then up-regulates the activity of NF-κB.
Significance: It was firstly found that the phosphorylation of Trx at T100 plays an important role in its cytoprotective activity in cancer cells. Thus, besides blocking the active site of Trx, inhibiting the phosphorylation of Trx at T100 may be a new potential cancer therapy target.

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Introduction

Thioredoxin (Trx), a 12 kDa protein, can protect organisms against various forms of cellular stress that lead to cell death (Li et al. 2009; Lu and Cederbaum 2007). Trx plays an important role in cell proliferation and cancer (Arner and Holmgren 2000). Furthermore, it functions as an anti-apoptotic factor because Trx inhibits apoptosis signal-regulating kinase 1 (ASK1), a critical factor involved in stress-induced cell death (Saitoh et al. 1998). Therefore, Trx is responsible for a line of drug resistances in cancer cells and is considered as a target for cancer therapy (Powis and Kirkpatrick 2007). However, disagreeing viewpoints indicate that over-expression of Trx could promote apoptosis via activation of caspase-8 (Ma et al. 2001; Dashnamoorthy et al. 2005). Consequently, detailed understanding of the functions of Trx is required before this protein can be used as a molecular target against cancer.

Modifications are found to regulate Trx activity. Besides the two cysteine residues located in the catalytic site, Trx harbors three other cysteines, C62, C69 and C73, which can be modified by ROS and nitric oxide (NO). For example, C73 reacts with glutathione and Y49 nitrosative modification resulting in the inhibition of Trx activity (Casagrande et al. 2002; Tao et al. 2006), while C69 nitrosylated by NO will enhance its redox regulatory activity (Haendeler et al. 2002). As such, the modifications induced by the balance of ROS and NO in cells may determine the activity of Trx.

Gevaert et al. identified 190 phosphorylated peptides from 152 different proteins, including Trx, in human HepG2 hepatocytes using reversed-phase diagonal LC (Gevaert et al. 2005). However, the role of phosphorylation in regulating Trx activity is unknown at present. In this study, the activity of the phosphorylated Trx at T100 as well as its possible mechanism was analyzed.

Materials and methods

Plasmid

The pGEM-Trx plasmid was constructed and conserved in our laboratory. It was used as the template to amplify a DNA sequence named 6His-Trx. The two primers are:

Forward, 5′-tcttgatcatatggtgaagcagatcgagag-3′,
Reverse, 5′-gtagtcgacttagtggtggtggtggtggtggactaattcat taatggtggcttc-3′.

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The 6His-Trx DNA fragment is cloned to the plasmid pcDNA3.1 and the recombined plasmid is named pcDNA-Trx. The QuikChange® site-directed mutagenesis kit (Merck) was used to obtain the plasmid pcDNA-mTrx which encoded the T100A mutant Trx using the following two primers (mutation bases are shown in capital letters):

Forward, 5′-cattaatgTtggcttcaagc-3′,
Reverse, 5′-gcttgaagccaAcattaatg-3′.

Cells

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone). The plasmids were extracted using an endo-free plasmid mini kit (Omega) according to the protocol provided by the manufacturer and were then stably transfected into the HepG2 cells using the Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. After 48 h, the transfection medium was changed to DMEM containing 10% FBS and 0.7 mg/ml G418 (Invitrogen). Individual colonies were obtained with G418 selection for 2 weeks. The colonies were grown and expanded in DMEM with G418 (0.4 mg/ml) for further assays.

6His pull-down assay to purify Trx-6His and mTrx-6His

About 10^7 HepG2 cells transfected with Trx-6His or mTrx-6His were collected and lysed with 1 ml Cell lysis buffer for Western and IP (Beyotime, China). The supernatant was incubated with 0.5 ml nickel–nitrilotriacetic acid Ni–NTA agarose (Pharmacia) at room temperature for 20 min, then washed 3 times with 1 ml phosphate buffer solution (PBS, 137 mM NaCl; 2.7 mM KCl; 100 mM Na_2HPO_4; 2 mM KH_2PO_4) for and twice with 1 ml Imidazole buffer (10 mM imidazole; 20 mM Tris pH7.9; 50 mM KCl; 10% glycerol; 1 mM PMSF). The Trx-6His or mTrx-6His proteins were eluted down with 0.5 ml Imidazole (100 mM imidazole; 20 mM Tris pH7.9; 50 mM KCl; 10% glycerol; 1 mM PMSF). This elution buffer was lyophilized into powder in a freeze dryer (Thermo). The powder was dissolved in 50 µl PBS and was used for Western blotting test. 10 mM $NaVO_3$ (Guoyao, China) was added to all of the buffers to inhibit the activity of phosphatase.

ELISA test

A sandwich enzyme-linked immunosorbent assay (ELISA) was used for phosphorylated Trx determination. The per well of ELISA plates (Costar) was coated with Trx monoclonal antibody (1.0 µg/ml) diluted in carbonate buffer, pH9.6, in a 200 µl volume and incubated for 1 h at 37 °C. After four washes with PBS containing 0.05% Tween20 (PBST), the plates were sealed with 250 µl BSA (10 mg/ml), and incubated for 1 h at 37 °C. After four washes with PBST, 20 µg total proteins extracted from HepG2 cells which were diluted in 200 µl PBST were added to the wells and incubated for 1 h. After four washes with PBST, 200 µl Phospho-Threonine Polyclonal Antibodies (0.5 µg/ml, New England Biolabs) were added to the wells and incubated for 1 h. After four washes with PBST, 200 µl Goat anti-rabbit secondary antibodies (1:20,000) were added to the wells and incubated for 1 h. After five washes, color was developed using 100 µl o-phenylenediamine dihydrochloride buffer (0.04% o-phenylenediamine dihydrochloride; 1.84% $Na_2HPO_4 \cdot 12H_2O$; 0.51% Sodium Citrate; 0.03% hydrogen peroxide). After 20 min, the reaction was stopped by the addition of 100 µl H_2SO_4 (2.0 M). The optical density (OD) of each well was read at 495 nm using a microplate reader (Sun-Rise).

Survival rate test

Approximately 3×10^3 HepG2 cells were treated with hydrogen peroxide (H_2O_2) in various concentrations in 96-well flasks. The medium was discarded and 100 µl of fresh medium and 10 µl Alamar Blue (SunBio, China) was added to each well. The cells were cultured in the dark for 4 h and the OD values at 570 nm were measured using a microplate reader (Sun-Rise).

Apoptosis detection

About 5×10^5 HepG2 cells in culture flasks were treated with 10 µM cis-platinum (CDDP, Sigma) or 100 ng/ml adriamycin (Adr, Sigma) for 20 h. The mediums were added with or without 10 nM staurosporine (Beyotime). Cells were washed twice with PBS, then incubated with 1 ml Hoechst33258 (Beyotime, China) at room temperature for 30 min. The medium was removed and the cells were washed twice with PBS. The presence of cells that had undergone apoptosis was observed under an inverted fluorescent microscope (Olympus).

Western blotting

About 1×10^6 cells were collected and resuspended in 50 µl PBS, then lysed by three freeze-thawing steps. The solution was centrifuged at 12,000 *g* for 10 min to yield the soluble protein fraction. The nuclear proteins were prepared using the Nuclear Fraction Kit (DBI) according to the manufacturer's instructions. About 20 µg proteins were separated using a 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). All proteins were subsequently blotted onto polyvinylidene fluoride (PVDF) membranes (Minipore) and immunoblotted with a monoclonal anti-ASK1 antibody (1 µg/ml, Ptglab), the Phospho-Threonine Polyclonal Antibody (2 µg/ml, New England Biolabs) or a 6His-tag antibody (1 µg/ml, Ptglab, China). A SuperSignal West Pico trial kit (Pierce) was used for chemiluminescence-based detection of the appropriate secondary antibodies.

Results

Trx mutated at T100 partly lost the ability to eliminate ROS induced by H_2O_2

Trx is well known to function as a scavenger of ROS which can be induced by H_2O_2. Therefore, we studied whether the mutation of Trx at T100 is involved in this function. We found that the survival rate of

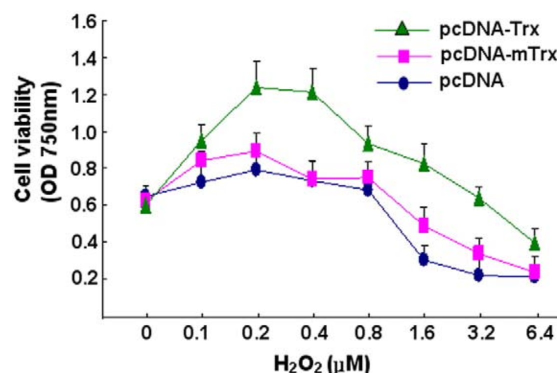

Fig. 1. HepG2 cell viability. The HepG2 cells transfected with pcDNA, pcDNA-Trx and pcDNA-mTrx were treated with H_2O_2 at various concentrations for 24 h. The survival rates were detected with Alamar Blue. Data shown are mean values ± SD (n = 4).

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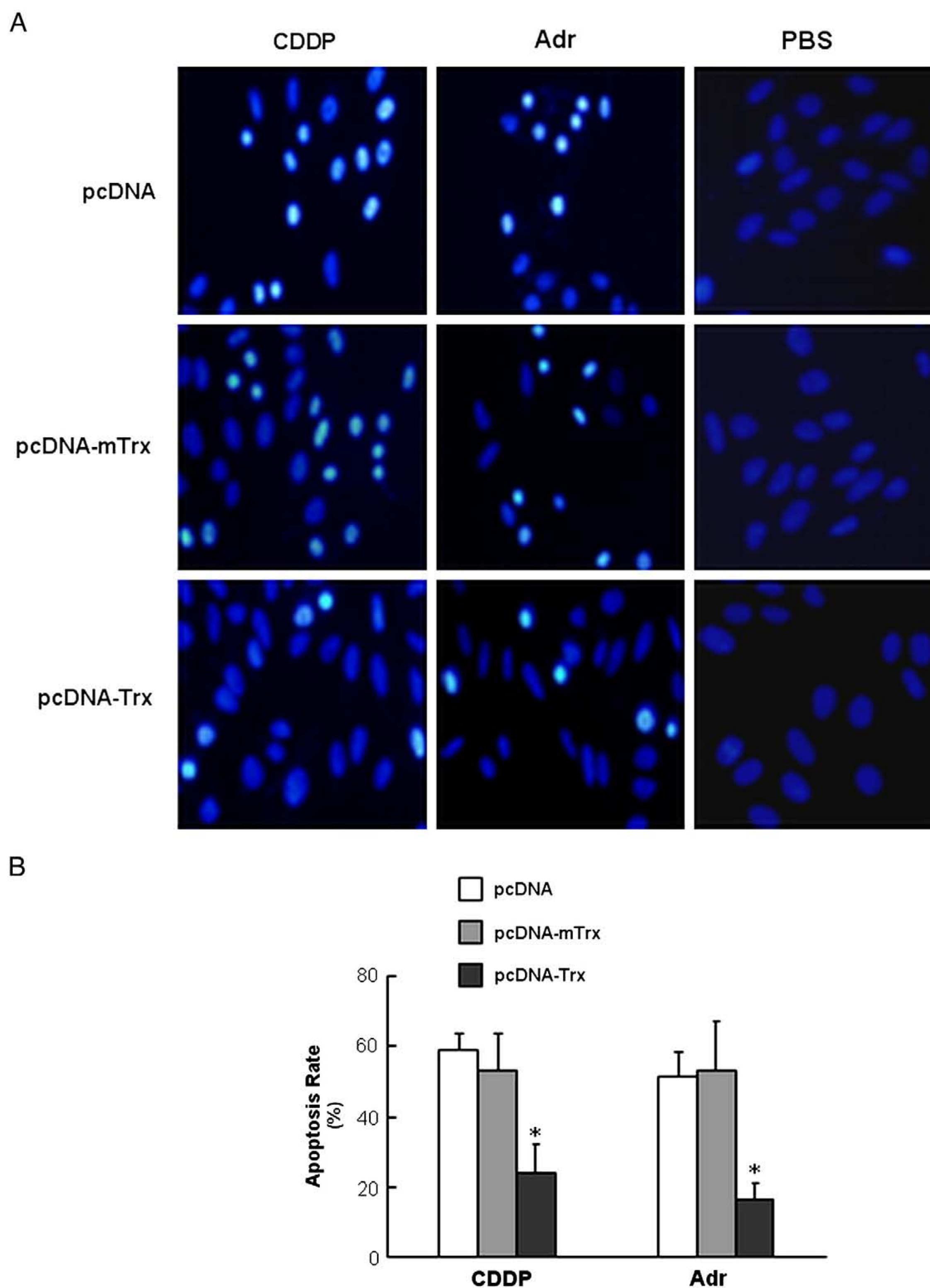

Fig. 2. The apoptosis detection of the HepG2 cells. HepG2 cells in culture flasks were treated with CDDP (10 μM) and Adr (100 ng/ml) for 20 h. The cells treated with PBS were used as controls. Cells were then stained with Hoechst33258 and observed under an inverted fluorescent microscope (A). The apoptosis rates were calculated based on A. Four different but equal visual areas were analyzed. Data shown are mean values ± SD (n = 4). The asterisk indicates statistical difference (*P<0.05) (B).

172 the HepG2 cells transfected with pcDNA-Trx was markedly higher
173 than the cells transfected with pcDNA-mTrx when they were treated
174 with H_2O_2 in gradient of concentrations (Fig. 1). The data indicate that
175 the T100 mutation of Trx reduces the ability of Trx to eliminate the
176 damage of ROS induced by H_2O_2.

mTrx offered less protection against CDDP or Adr-mediated apoptosis 177
than Trx 178

It is well known that Trx contributes to the drug resistance in 179
cancer therapy. To further explore the importance of T100 residue in 180

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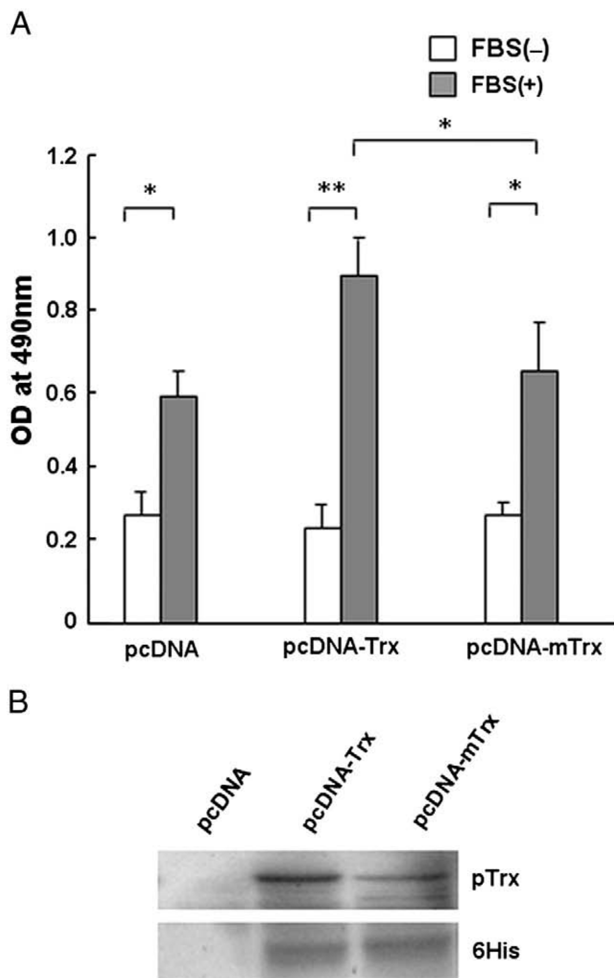

Fig. 3. The levels of phosphorylated Trx in HepG2 cells. Cells were starved overnight and treated with or without 10% fetal bovine serum for 1 h. Cells were then lysed and the total proteins (20 μg) were used to analyze the phosphorylation state of Trx by ELISA. The means ± SD (n = 3) are shown. The asterisk indicates statistical difference (*P<0.05, **P<0.01) (A). The proteins were prepared with the 6His pull-down method used for Western blot experiments. The primary antibodies were the phospho-Threonine antibody and 6His-tag antibody (B).

Trx, we also detected the rate of apoptosis in the HepG2 cells after they were treated with CDDP or Adr for 24 h. It was found that, compared with the cells transfected with Trx, the cells transfected with mTrx showed higher rate of apoptosis when they were treated with the two drugs (Fig. 2). This result suggests that the T100 residue mediates the protective effect against cancer drugs.

Trx can be phosphorylated at T100

Trx has previously been suggested to be phosphorylated at T100 (Gevaert et al. 2005) and the evidence in our study supports this. When the HepG2 cells transfected with pcDNA, pcDNA-Trx or pcDNA-mTrx were treated with or without FBS, their total proteins were used to detect the levels of phosphorylated-Threonine by ELISA. It was found that more Trx was recognized by the anti-phosphorylated-T antibody in the cells treated with FBS, compared with that without FBS. Thus, it is clear that T(s) of Trx can be phosphorylated. However, the levels of phosphorylation on Trx in the pcDNA-mTrx or pcDNA transfected cells rose about 2-fold, and that in the pcDNA-Trx transfected cells rose more than 3-fold (Fig. 3A). When the exogenous Trx, which expressed by pcDNA-Trx or pcDNA-mTrx in the cells treated with FBS, were purified by Ni–NTA and detected by Western blot, less phosphorylated Trx can be detected in the pcDNA-mTrx transfected cells than that in the pcDNA-Trx transfected cells (Fig. 3B). These data showed clearly that the T100 of Trx can be phosphorylated in HepG2 cells.

PKC is involved in regulating the phosphorylation of Trx

It has been shown that protein kinase C (PKC) phosphorylates and modify the actions of its downstream targets. Next, we explored whether the T100 of Trx is a target of PKC. When the HepG2 cells were treated with or without phorbol-12-myristate-13-acetate (PMA), a activator of PKC, the levels of phosphorylated Trx were sharply increased compared to the levels of phosphorylation of the T100A mutant Trx (Fig. 4A). Furthermore, when phosphorylation was induced by CDDP or Adr in the presence of staurosporine, an inhibitor of PKC, the pcDNA-Trx transfected cells that were apoptotic were as many as those that were apoptotic in the pcDNA-mTrx transfected cells line (Fig. 4B and C). Therefore, it can be concluded that the T100 of Trx is a downstream target in the PKC pathway. These data also directly proved that phosphorylation on Trx at T100 can markedly enhance its anti-apoptotic function.

Phosphorylated Trx enhances the activity of NF-κB, but does not influence Trx-ASK1 complex levels

Next, we studied how the phosphorylated Trx takes part in the apoptosis pathway. ASK1 is preferentially activated by ROS and is required for apoptosis (Tobiume et al. 2001). Trx is known to inhibit the kinase activity of ASK1 by directly interacting with the N-terminal region of ASK1 (Saitoh et al. 1998). The total Trx-6His or mTrx-6His proteins in the HepG2 cells transfected with pcDNA, pcDNA-Trx or pcDNA-mTrx could be purified by Ni–NTA. SDS-PAGE electrophoresis and Western blotting analysis was then performed for detecting Trx-6His and ASK1. As shown in Fig. 5A, the complex level of mTrx-ASK1 was same as that of Trx-ASK1. Therefore, it can be concluded that the phosphorylation at residue T100 of Trx does not regulate the formation of the Trx-ASK1 complex. It also well known that the nuclear Trx can enhance the activity of NF-κB (Hirota et al. 1999). When the nuclear proteins were extracted, the proteins 6His-Trx and 6His-mTrx were purified through 6His-pull-down and they were detected by Western blotting. It was found that with the absence of PMA, there was no difference in the levels of nuclear Trx between the pcDNA-mTrx and pcDNA-Trx transfected cells; in the presence of PMA, more nuclear Trx were detected in the cells transfected with either pcDNA-mTrx or pcDNA-Trx. However, smaller rate of increase in nuclear Trx was found in the cells transfected with pcDNA-mTrx than that of pcDNA-Trx. Meanwhile, greater rate of increase in nuclear p65 was found in the cells transfected with pcDNA-Trx than that of pcDNA-mTrx when the cells were treated with PMA, though PMA stimulation resulted in more p65 locating into the nucleus of the cells transfected with either pcDNA-mTrx or pcDNA-Trx (Fig. 5B). These data indicated that the phosphorylation contributes to the Trx translocation into the nucleus and subsequent enhancing the activity of NF-κB.

Fig. 4. Effects of PKC activator or inhibitor. The HepG2 cells transfected with pcDNA-Trx or pcDNA-mTrx, with or without PMA (1 μM) stimulation for 20 h, were used for ELISA to test the levels of phosphorylated Trx. The means ± SD (n = 3) are shown. The asterisk indicates statistical difference (*P<0.05) (A). HepG2 cells transfected with pcDNA-Trx or pcDNA-mTrx were treated with CDDP (10 μM) and Adr (100 ng/ml) for 20 h. The mediums were added with or without staurosporine (10 nM). Cells were then stained with Hoechst33258 and observed under an inverted fluorescent microscope (B). The apoptosis rates were calculated based on B. Four different but equal visual areas were analyzed. Data shown are mean values ± SD (n = 4). The asterisk indicates statistical difference (*P<0.05) (C).

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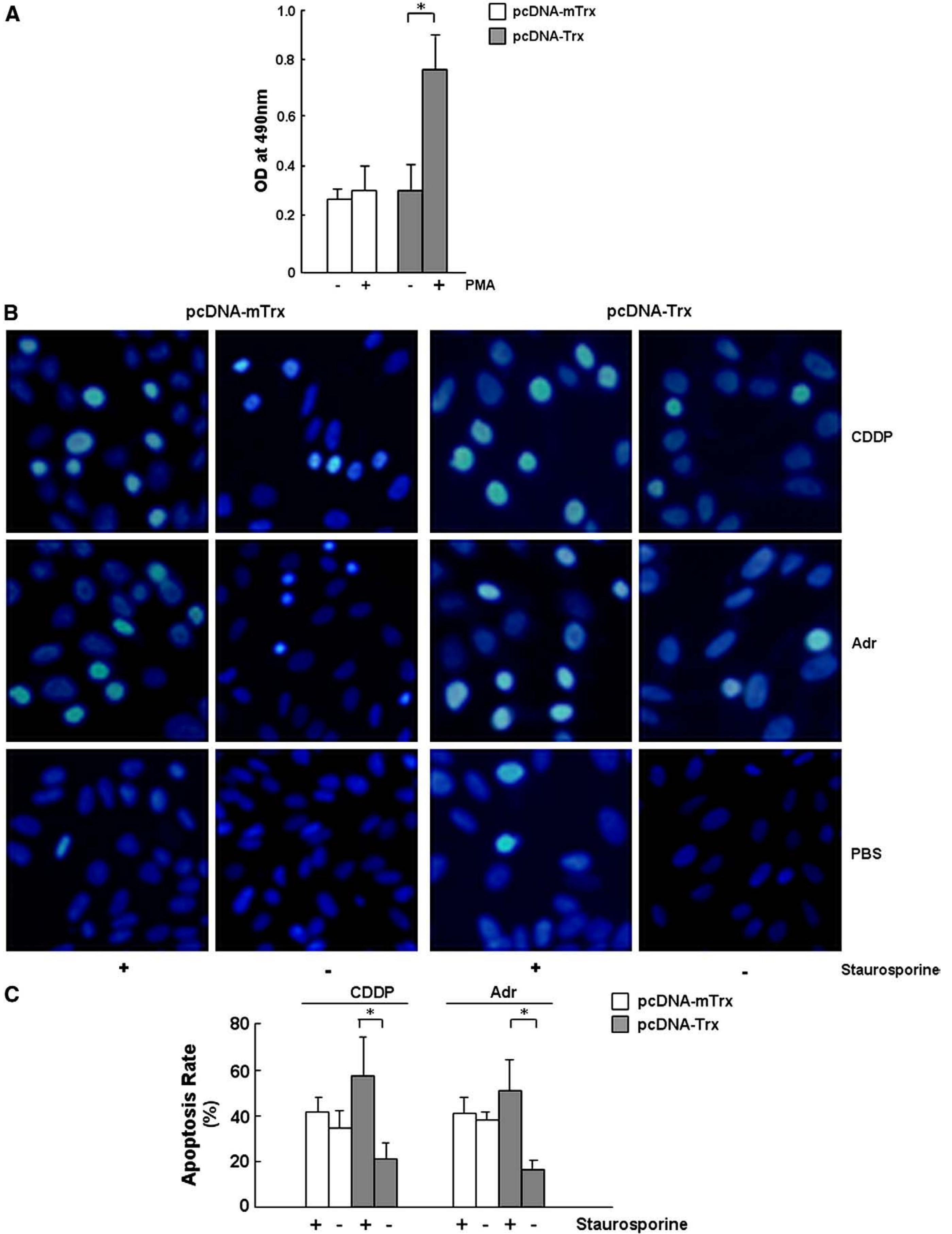

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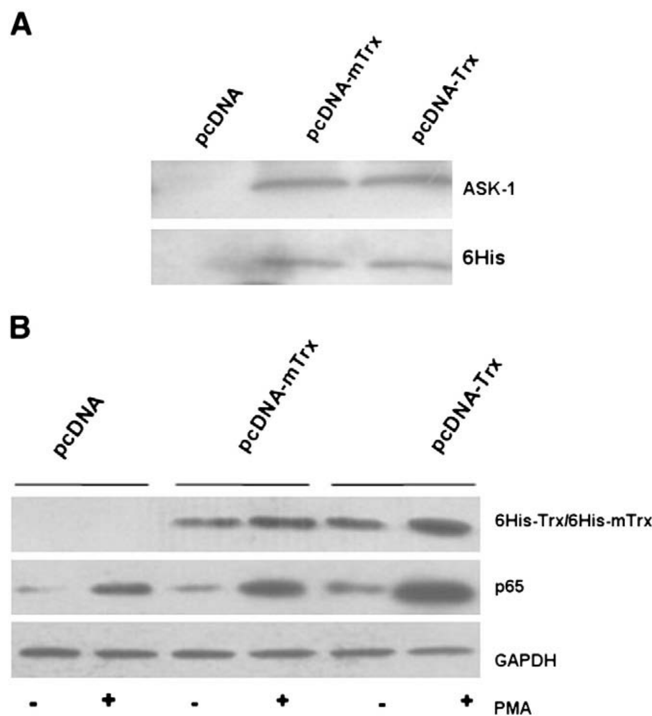

Fig. 5. The levels of the Trx-ASK1 complex and the detection of p65. The total Trx-6His or mTrx-6His proteins in the transfected cells were purified with Ni–NTA agarose. The eluted solution was separated with SDS-PAGE and the levels of Trx-6His and ASK1 were detected by Western blotting (A). HepG2 cells were treated with (+) or without (−) PMA (1 μM) for 20 h and the nuclear proteins were extracted and used for western blotting to detect the levels of p65 and GAPDH. The nuclear Trx prepared with the 6His pull-down method was used for Western blot to detect the levels of 6His-Trx or 6His-mTrx (B).

Discussion

This study presents evidence that the phosphorylation of Trx at T100 is needed for its activity to reduce the efficacy of cancer drugs in HepG2 cancer cells. Although the active site of Trx (C32 to C35) is crucial to the function of this protein, the modification of other residues can also profoundly enhance or suppress the activity of Trx (Haendeler et al. 2002; Tao et al. 2006). In this study, phosphorylation of residue T100 of Trx was again proved in HepG2 cancer cells. Trx with mutated T100 decreases the ability of HepG2 cells to survive against drug toxicity. Trx has been proposed to act as a factor that protects cancer cells from apoptosis induced by several drugs including CDDP (Arnold et al. 2004), but not Adr (Kawahara et al. 1996). Surprisingly, the mutant Trx appeared to furnish HepG2 cells Trx with enhanced sensitivity to CDDP and Adr (Fig. 2). Thus, the phosphorylated T100 of Trx protects cancer cells against cytotoxic cancer drugs. It is interesting that other Threonine (s) of Trx also can be phosphorylated (Fig. 3) and additional study is needed to explore their functions.

Phosphorylation of proteins plays a significant role in a wide range of cellular processes. Phosphorylation of proteins can introduce conformational changes in the structure of proteins via interaction with other hydrophobic and hydrophilic residues. In this study, the mutant T100 of Trx was found not to influence the levels of the Trx-ASK1 complex formed. Only the active site, Cys32 and Cys35, of reduced Trx is known to interact with ASK1 (Powis and Kirkpatrick 2007). Probably, the phosphorylation of Trx at T100 is distal from the active site and has no direct influence on the structure around the active site. However, T100 is located between Cys62 and Cys69 in the three-dimensional structure. ROS stress induces reduced Trx to convert into an oxidized form with the formation of the disulfide bonds. A second disulfide bond is formed between Cys62 and Cys69 when Trx is further oxidized (Watson et al. 2003). The phosphorylation of Trx at T100 may influence the disulfide bond formation between C62 and C69, which is important for Trx to regulate the DNA binding activity of NF-κB (Matthews et al. 1992). This supposition can be partly supported by the data in this study (Fig 5B).

Trx functions both in cytoplasm and in nucleus, through inhibiting IκB kinases and up-regulating NF-κB transcriptional activities, respectively (Hirota et al. 1999). The nuclear location of Trx will be more effective in reducing apoptosis than in cytoplasm, at least in some special circumstances (Chen et al. 2007). Though thioredoxin-interacting protein (TBP) was thought to mediate the nuclear localization of Trx (Nishinaka et al. 2004; Yoneda 2000), the mechanism of its nuclear transfer is still unknown. Phosphorylation of proteins may play a key role for their translocation into the nucleus (Xuan et al. 2006). Recently, we have reported that the levels of nuclear Trx were not enhanced in the cells transfected with pcDNA-Trx (Chen et al. 2007). In this study, though the enhanced nuclear Trx can be detected both in the pcDNA-mTrx transfected cells and in the pcDNA-Trx transfected cells when they are treated with PMA, a PKC activator, smaller increase in nuclear Trx was found in the cells transfected with pcDNA-mTrx than that of pcDNA-Trx. These data indicated that the phosphorylation contributes to the nuclear translocation of Trx. Therefore, additional study is needed to explore whether the phosphorylation of T100 is required for Trx to bind with TBP.

Conclusion

In summary, we have found that the ability of Trx to protect HepG2 cells against anticancer drugs is mediated by phosphorylation at T100 site by a PKC-dependent pathway. Previously, drugs targeting the active site (C32–C35) were designed to treat cancer (Mukherjee et al. 2005). Our observations in this study raise the interest on whether inhibiting the phosphorylation of Trx represents a potential cancer therapy target.

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “Thioredoxin-1 phosphorylated at T100 is needed for its anti-apoptotic activity in HepG2 cancer cells”.

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