学位論文の要旨

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学位論文名	Thioredoxin2 Enhances the Damaged DNA Binding Activity of mtTFA Through Direct Interaction
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論文内容の要旨

INTRODUCTION

The cytotoxic action of cisplatin is believed to result from the formation of covalent adducts with DNA. Cisplatin-targeted sequences, such as G-stretch sequences, appear much more frequently in mtDNA than in nuclear DNA. Thus, we propose that mtDNA might be the main target of cisplatin. mtDNA is more susceptible to oxidative damage than genomic DNA because of its lack of a nucleosome structure. Mitochondrial transcription factor A (mtTFA) is a member of the HMG (high mobility group)-box protein family, members of which stimulate transcription by binding to the D-loop region of mtDNA and function in mtDNA maintenance and repair. mtTFA also preferentially recognizes oxidatively damaged DNA as well as cisplatin-damaged DNA. The enhanced binding affinity of mtTFA for damaged DNA may suggest that mtTFA protects mtDNA from various DNA damage. We found that the expression levels of both mtTFA and TRX2 are upregulated in cisplatin-resistant cells. We also investigated the physical and functional interaction between mtTFA and TRX2.

MATERIALS AND METHODS

Cell culture. Human epithelial cancer HeLa cells were cultured. Cisplatin-resistant HeLa/CP4 cells were derived from HeLa cells.

Plasmid construction. To obtain the full-length cDNA for human TRX2, PCR was carried out on a SuperScript cDNA library using the primer pair. The PCR product was cloned into the pGEM-T easy vector. To construct Flag-tagged TRX2 expression plasmid in bacteria, the *EcoRI* fragment of TRX2 cDNA was ligated into the TH-Flag vector. For construction of pcDNA3-Flag-TRX2, N-terminal Flag-tagged TRX2 cDNAs were ligated into a pcDNA3 vector. The construction of GST-mtTFA, GST-mtTFAΔ1.2, GST-mtTFAΔ2, and GST-mtTFAΔ1 has been described previously. GST-mtTFA-CC (wild-type), -GC (cysteine 49 to glycine), -CX (cysteine 246 to stop codon), and –GX (cysteine 49 to glycine and cysteine 246 to stop codon) were obtained by PCR using the primer pairs.

Expression and purification of GST-fusion proteins. GST fusion proteins induced by 1 mM isopropyl-β-D-thiogalactopyranoside were sonicated in binding buffer, and soluble fractions were mixed with glutathione-Sepharose 4B. GST-fusion proteins eluted with 50 mM Tris-HCl and 20 mM reduced glutathione according to the manufacturer's protocol were separated in SDS 10% polyacrylamide slab gels. The eluted proteins were dialyzed in dialysis buffer using a PlusOne Mini DialysisKit.

Co-immunoprecipitation assay. 2×10^5 HeLa cells were seeded into 35-mm tissue culture plates. The following day, cells were transfected with 1 μg of each of HA- and Flag-fused expression plasmids using SuperFect reagent. At 6 h post-transfection, the cells were washed with PBS, cultured at 37 °C for 48 h in fresh medium and then lysed in buffer X. The lysates were centrifuged at 21,000 × *g* for 10 min at 4 °C and supernatants (300 μg) were incubated for 2 h at 4 °C with anti-Flag affinity gel. Immunoprecipitated samples were washed three times with buffer X and subjected to subsequent Western blot analysis.

GST pull-down assay. GST-mtTFA or its deletion mutants immobilized on glutathione-sepharose 4B were incubated with soluble bacterial extracts containing Flag-TRX2 for 2 h at 4 °C in buffer X. Bound samples were washed three times with buffer X and subjected to Western blotting analysis with anti-Flag antibody.

Western blot analysis. The indicated amounts of whole-cell lysates and nuclear extracts or immunoprecipitated samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride microporous membranes using a semidry blotter. The following primary antibodies were used: anti-mtTFA, anti-TRX2, anti-β-actin, and anti-Flag. Membranes were then incubated for 45 min at room temperature with a peroxidase-conjugated secondary antibody or a 1:5000 dilution of anti-HA-peroxidase. Bound antibody was visualized using an enhanced chemiluminescence kit and membranes were exposed to film.

Electrophoretic mobility shift assay. The following annealed 22-mer duplexes were prepared: 5'-GGTGGCCTGACXCATTCCCCAA-3' and 3'-ACCGGACTGYGTAAGGGGTTGG-5', where X = G or 8-oxo-dG and Y = A, C, G or T. Duplexes were end-labeled with [α-^{32}P]dCTP using the Klenow fragment for extension, and gel-purified. Half the volume of the labeled oligonucleotide without 8-oxo-dG was treated with 0.3 mM cisplatin at 37 °C for 12 h, and then purified by ethanol precipitation. Purified GST fusion proteins were used in EMSAs. Reaction mixtures contained 5% glycerol, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.4 ng/μl of ^{32}P-labeled probe DNA and the indicated amounts of GST fusion proteins, and were mixed. Binding reactions were incubated for 5 min at room temperature. Products were analyzed on 4% polyacrylamide gels in 0.5 X Tris-borate EDTA buffer using a bioimaging analyzer.

RESULTS AND DISCUSSION

p53 interacts with mtTFA and enhances the DNA binding activity of mtTFA to cisplatin-damaged DNA. Cisplatin induces both DNA damage and oxidative stress in mitochondria and thioredoxin1 is upregulated in cisplatin resistant cells. Based on these previous results, we examined the expression of mtTFA and TRX2 in cisplatin-resistant cells. The expression levels of both mtTFA and TRX2 were upregulated in cisplatin-resistant cells.

mtTFA interacts with TRX2 in vivo. To confirm the association and binding site, we performed a pull-down assay using immobilized GST-fusion proteins comprising mtTFA deletion mutants and Flag-TRX2. The HMG box 1 motif of mtTFA directly participates in the interaction.

The HMG box 1 motif possesses damaged DNA binding activity, but that the HMG box 2 motif does not. The interaction of mtTFA with TRX2 may alter the damaged DNA binding activity of mtTFA. An EMSA showed that GST-mtTFA can form a specific complex with cisplatin-damaged DNA and oxidized DNA. Addition of TRX2 to the mtTFA–DNA binding reaction resulted in significant enhancement of binding of mtTFA to both cisplatin-damaged and oxidized DNA.

mtTFA formed multimers under physiological conditions. To confirm whether the interaction of TRX2 with mtTFA requires a specific structure of mtTFA, we introduced mutations at two cysteine residues, positions 49 and 246. Pull-down assays showed that two mtTFA mutants, GC and CX, interacted with TRX2 with the same affinity as CC. However, the association of mtTFA with TRX2 was significantly enhanced when the GX mutant was used. This indicates that the cysteine-dependent secondary structure of mtTFA may regulate its association with TRX2. Oxidized DNA binding was increased when the GX mutant was used. The enhancement of the DNA binding activity of mtTFA by TRX2 was observed for all mutants.

We observed a significant increase in the levels of the mtTFA and TRX2 in cisplatin-resistant cells. These data suggest a functional interaction between mtTFA and TRX2 because both proteins are localized in mitochondria. Mitochondria function as central players in modulating apoptosis. Little is known regarding the function of TRX2 and its potential role in apoptosis. Our data also reveal that TRX2 expression is upregulated in cisplatin-resistant cells, consistent with the fact that drug-resistant cells often show apoptosis-resistant phenotypes. Additionally, significantly increased levels of apoptosis have been observed in mtTFA-knockout mice. mtTFA functions not only as a transcription factor, but also as DNA binding protein to protect mtDNA.

Both HMG box 1 and 2 motifs could interact independently with p53, which is localized to mitochondria under conditions of DNA damage stress. TRX2 interacts with the HMG box 1 motif of mtTFA. This suggests that the secondary structure of mtTFA may modulate its association. We introduced mutations at two cysteine residues of mtTFA. The association of TRX2 with mtTFA was enhanced when the GX mutant was used. TRX2 can support DNA binding by mtTFA, even when a conformational change in mtTFA is induced by ROS, suggesting that mtTFA can still bind and protect mtDNA under conditions of oxidative stress. The physiological multimerization of mtTFA may efficiently support DNA binding to mtDNA. A conformational change in mtTFA might inhibit multimerization. Therefore, TRX2 can enhance the DNA binding activity of damaged mtTFA, which cannot form multimers, to protect mtDNA.

CONCLUSION

In this study, we identified a novel interaction of mtTFA with TRX2. TRX2 supports the DNA binding activity of mtTFA to damaged DNA through a direct interaction. Our findings suggest that TRX2 not only functions as an antioxidant defense, but also cooperatively acts to support mtTFA functions. Characterization of the interaction of TRX2 with mtTFA will aid our understanding of the mtTFA function in both normal and pathological conditions.