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Interaction of metallothionein with tumor suppressor p53 protein

Elena A. Ostrakhovitch[a,*], Per-Erik Olsson[b], Sean Jiang[a], M. George Cherian[b]

[a] *Department of Pathology, University of Western Ontario, London, Ont., Canada N6A 5C1*
[b] *Örebro Life Science Center, Department of Natural Science, Örebro University, Örebro, Sweden*

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Abstract **Previous reports have shown that metallothionein (MT) may modulate p53 activity through zinc exchange. However, little is known on a direct interaction between MT and p53 in cells. The results demonstrate an interaction between MT and p53 can occur in vitro. The complex between MT and p53 was observed in breast cancer epithelial cells with both wild and inactive type of p53. Furthermore, it was shown that wt-p53 was preferentially associated with Apo-MT. Our data suggest that co-expression of MT and p53 and their complex formation in tumor cells may be involved in regulation of apoptosis in these cells.**

Keywords: p53; Metallothionein; Protein interaction

1. Introduction

Metallothionein (MT) is a family of conserved metal-binding proteins with a potential role on homeostasis of essential metals and intracellular storage of metal ions. The affinity of zinc ions to cysteines in MT provides an efficient mode of sequestration of zinc, and thus, provides an intracellular source of zinc for a number of proteins and enzymes such as, estrogen receptor, transcription factor IIIa and mitochondrial aconitase [3,6,9]. MT was found to interact with the p50 subunits of NF-κB, kinase domain of PKCμ and GTPase Rab3A [1,10,16]. While high expression of MT in certain tumors may block apoptosis and be related to tumor progression, a central role for p53 is to act as a tumor suppressor by inducing apoptosis [4]. The tumor suppressor p53 is a transcriptional factor with a key role in maintenance of genome integrity, regulation of cell cycle and apoptosis. Recent studies have indicated a strong relationship between p53 and MT since high expression of MT in tumors is consistently associated with the presence of mutated p53 and increased tumor grade. It has been suggested that MT can regulate the DNA binding activity of p53 through zinc transfer reaction [12]. The zinc ions are essential for maintenance of the wild-type conformation and stability of p53 protein and its affinity for specific DNA sequence for transcriptional activity of the protein [13,15]. However, transfer of zinc can also occur in the reverse direction. The metal-free form of MT (apo-MT) with its 20 sulfhydryl groups can sequester zinc and thereby, reduce the transcriptional activity of p53 [12]. Release of zinc ion from p53 results in a highly aggregation prone conformation [2]. Although MT has been implicated in the control of p53 folding with zinc exchange, there is little information on direct interaction between MT and p53 proteins. The biological activity of p53 protein is mainly related to it transcriptional functions, but it has been suggested that p53 can exert its function via protein–protein interaction by binding to partner proteins such as c-Abl, Ref. [1], F-actin, and Bax [5,7,8].

This study was undertaken to examine a potential direct interaction of p53 and MT. We show that p53 can be associated with MT both in vivo and in vitro. In vitro, wt-p53 associates preferentially with apo-MT.

2. Materials and methods

2.1. Cell culture

Human breast cancer epithelial cell line MN-1, containing wt-p53 and the MDD2 cell line, a variant derived from MCF-7 by transfection with inactive p53 (pCMVDD-p53; mut-p53), were generously provided by Dr. C.M. Galmarini, (Laboratoire de Cytologie Analytique, Faculté de Médecine Rockefeller, France). Both cell lines were originally made by M. Oren [14] and maintained in complete Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin).

2.2. Protein preparation

The Zn, Cd-MT was prepared from rabbit livers as described [18]. The glutathione *S*-transferase (GST)-tagged wt-p53 protein was isolated from a strain of *E. coli* that contained the coding sequence of wild type human p53 (purchased from Protein One, Technology Dive, College Park, MD 20742).

Apo-MT was prepared by gel-filtration chromatography (Sephadex G-50) at pH 2, as described by Vasak [19]. Metal-free protein was prepared one day before the experiments and was maintained at pH 2. To avoid metal contamination, deionized water and metal-free pipette tips were used throughout. Zinc and cadmium contained in MT was analyzed by flame atomic absorption spectroscopy. Protein concentrations of MT and fractions from Sephadex G-50 gel-filtration column were determined by spectrophotometry ($\varepsilon_{220} = 48\,200\ M^{-1}\ cm^{-1}$ [19].

2.3. Circular dichroism (CD) spectroscopy

CD spectra were recorded using a J-810 spectropolarimeter (Jasco) in 10 mM sodium phosphate or 20 mM Tris–HCl (pH 7.4) at 20 °C in 0.1 cm quartz cuvette for far-UV CD spectroscopy. Concentration of protein was 100 μg/ml. CD spectra were recoded in a spectral range of 190–260 nm at 25 °C. Molecular elipsicity (Θ) was expressed as deg cm^2 $dmol^{-1}$.

2.4. Co-immunoprecipitation and Western blot analysis

MN1 cells and MDD2 cells were treated with 100 μM of $ZnSO_4$ for 14 h, lysed in lysis buffer and centrifuged for 15 min at 12000×g. The protein concentration was determined using a detergent-compatible

*Corresponding author. Fax: +1 519 6613370.
E-mail address: eostrakh@uwo.ca (E.A. Ostrakhovitch).

Abbreviations: MT, metallothionein; CD, circular dichroism

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Bio-Rad protein assay (Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario). The 100 μl of cell extract (5 mg/ml) was immunoprecipitated with 10 μl of monoclonal anti-MT (Dako Cytomation Inc., Mississauga, Canada) or polyclonal anti-p53 antibodies (clone FL-393, Santa Cruz, CA). Then, protein A/G-sepharose beads were added to the immunoprecipitates and mix overnight at 4 °C. The beads were washed 4–5 times with lysis buffer, boiled in SDS sample buffer, fractionated by SDS–PAGE, and blotted with the indicated primary antibodies.

For Western blot analysis, the whole cell extracts were normalized for protein and 60 μg of each cell lysate was separated by 12% SDS–PAGE and blotted with anti-p21 (Santa Cruz, CA) and anti-MT antibodies, followed by HRP-conjugated secondary antibodies. Loading of equal amount of protein was confirmed by blotting with anti-GAPDH antibody (HyTest Ltd., Turku, Finland). The bands were visualized by enhanced chemiluminescence, using ECL reagent and ECL Hyperfilm (Amersham, Que., Canada). All experiments were conducted at least three times.

2.5. GST-pull-down assay

The 100 μg of GST-tagged p53 protein was attached to glutathione-Sepharose beads and incubated with purified rabbit liver MT1 in binding buffer (ProFound pull-down GST protein–protein interaction kit, Pierce Biotech, Rockfold, IL). The proteins were then separated on 12% gel by SDS–PAGE, followed by Western blot analysis using anti-p53 or anti-MT antibodies.

3. Results

3.1. P53 can interact with MT

In epithelial breast cancer MN1 cells derived from MCF-7 cells with wt-p53, the basal levels of MT and p21 (the downstream target of p53) were low (Fig. 1A). Treatment of MN1 cells with zinc (100 μM) resulted in increase in p21 protein level, suggesting the cellular accumulation of functional p53. The elevated p21 expression is mainly associated with stabilization and accumulation of functionally active p53. In addition, as expected there was an increase in MT levels after zinc exposure (Fig. 1A). Unlike MN1, the basal level of MT was high in epithelial breast cancer MDD2 cells with a inactive form of p53. Treatment of MDD2 cells with zinc did not significantly change the level of either MT or p21, suggesting a relationship between expression of p53 and MT in these cells derived from MCF-7 cells. In order to determine if p53 interacted with MT, we examined the p53-MT complex formation in MN1 and MDD2 cells with and without treatment with zinc (Fig. 1B and C). Using monoclonal anti-MT antibody, p53 protein was co-precipitated with MT from the protein lysates of both MN1 cells and MDD2 cells (Fig. 1B). Furthermore, using polyclonal anti-p53 antibody we also observed co-precipitation of MT protein in both MN1 and MDD2 cells (Fig. 1C). These results indicate that p53 and MT can form a complex in epithelial breast cancer cells, irrespective of their p53 status.

3.2. The structural characterization of MT-p53 interaction

Various fractions of rabbit liver MT with different metal content were collected from gelfiltration chromatography. The UV/CD spectra of apo-MT and metal containing MT confirmed their structural properties. The fraction with spectrum displayed a minimum at 200 nm contained mainly apo-MT (Fig. 2A).

To determine the nature of the p53-MT interaction, we employed a GST-pull-down assay to analyze the in vitro GSH-wt-p53 interaction with purified rabbit liver Cd, Zn-MT1 and apo-MT1 as described in Fig. 2B. Unbound proteins

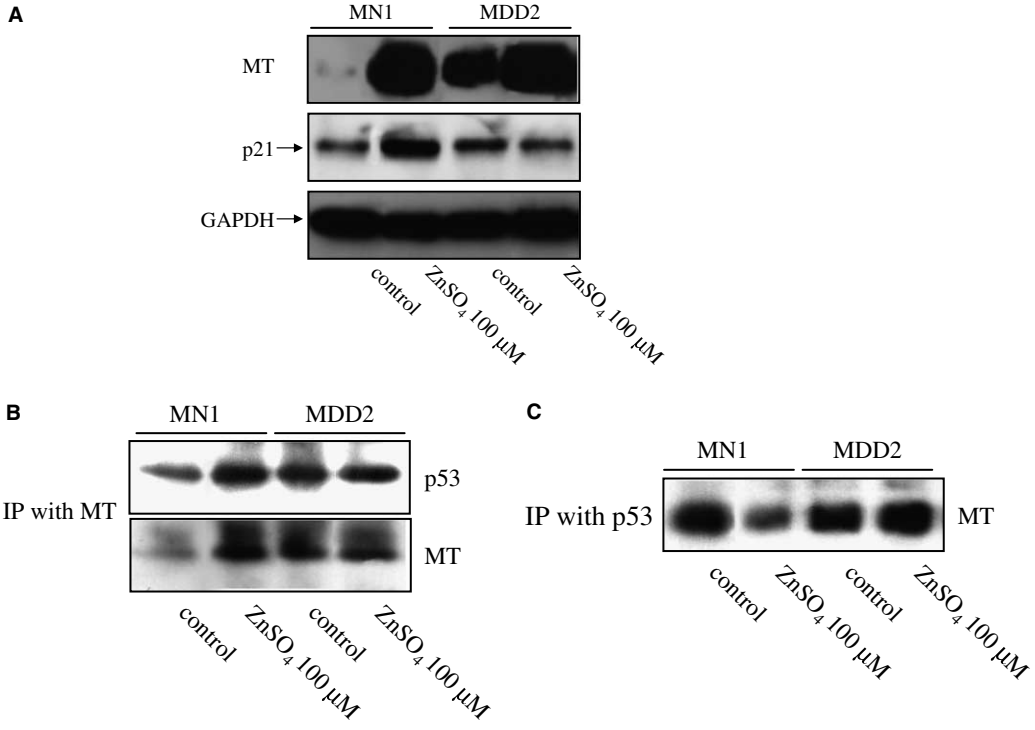

Fig. 1. (A) Induction of MT and p21, a downstream target gene of p53, by zinc. Immunoblots of whole-cell extracts, from MN1 and MDD2 cells treated with 100 μM of $ZnSO_4$ for 14 h, were examined for MT and p53 interaction. Co-immunoprecipitation of p53 and MT was examined in MN1 and MDD2 cells treated with or without 100 μM of $ZnSO_4$ for 14 h. The cell extracts were immunoprecipitated (IP) with monoclonal anti-MT antibody (B) or with polyclonal p53 antibody (C). The immunoprecipitates were probed with antibodies to MT or p53.

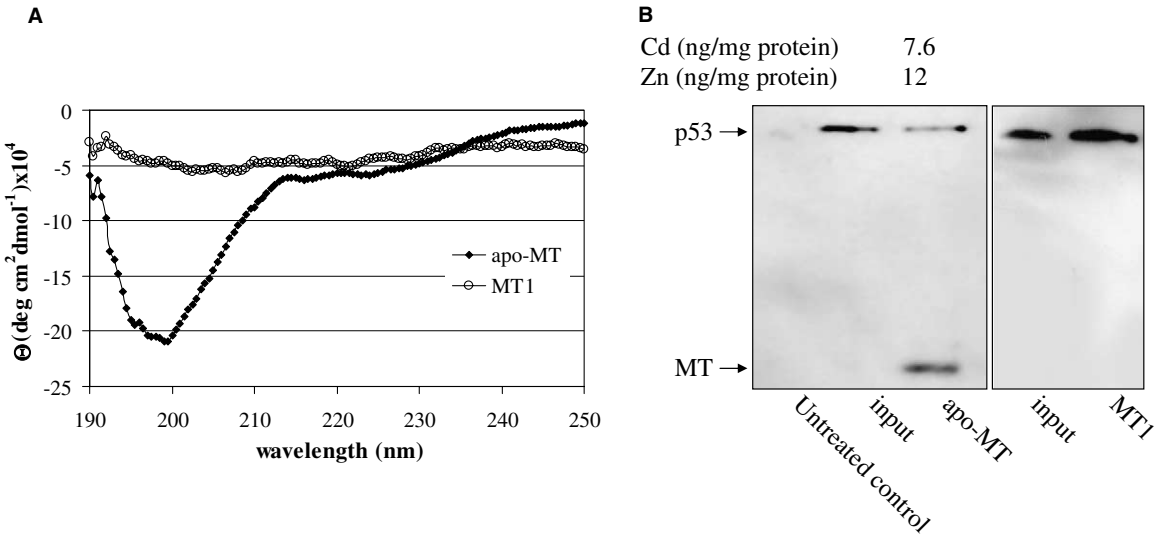

Fig. 2. (A) CD profile of Cd, Zn-MT and apo-MT after gel filtration at pH 7.4. (B) p53 binds to apo-MT, but not to Cd, Zn-MT. GST-pull-down analysis. Aliquots of purified rabbit liver Cd, Zn-MT1 and apo-MT1, prepared by gel-filtration chromatography were incubated with GST-wt-p53 bound to glutathione-Sepharose. Protein complexes were analyzed by SDS–PAGE. Input was the positive control with p53 alone.

were removed by washing and the bound proteins were visualized by enhanced chemiluminescence. We found that only apo-MT was able to bind to immobilized GST-wt-p53. In this in vitro assay, the interaction of purified Cd, Zn-MT with wt-p53 was not detected. Overall, these data indicate that p53 can bind directly with apo-MT and not with MT.

4. Discussion

Although, it is reported that MT can modulate biological activity of p53 via zinc exchange, there is no direct evidence that zinc exchange occur through direct interaction between MT and p53. In order to investigate the possibility of direct interactions of MT with p53, we performed immunoprecipitation using antibodies to p53 or MT on cell lysates of epithelial breast cancer cells with different p53 status; MN1 cells with wt-p53 and MDD2 cells with inactive p53. Both these cell lines were derived from human breast cancer epithelial MCF-7 cells [14]. Exposure of MN1 cells to zinc resulted in induction of p53-dependent transcription of p21 and increase in expression of MT. In these cells, an immune complex between MT and p53 was observed when p53 was precipitated with anti-MT antibody and vice versa. Thus both antibodies to p53 and MT were able to bring down the MT-p53 complex. It is interesting that the complex formation between MT and inactive p53 was also observed in MDD2 cells with high basal level of MT. Together these data suggest that MT-p53 complex exists in the cells.

The metal-free form of MT (apo-MT) is usually only present in the cell, when it is transiently generated during protein synthesis. It has been shown that apo-MT can regulate the folding of zinc metalloproteins including the p53 by competing for zinc [3,11,12,17,21]. It has also been shown, that apo-MT can disrupt the DNA binding activity of TFIIA, Zn-Sp1, and p53 through zinc chelation [12,20,21]. However, a recent report showed the presence of apo-MT in certain tumor cells and direct biomolecular reactions between apo-MT and the Zn-finger sites of TFIIIa [9]. These data are consistent with our results based on of the GST-pull-down assay, which demonstrate that only apo-MT1, and not MT1, forms a complex with p53. The complex might be formed by interaction between sulfhydryl groups of apo-MT and zinc ion of p53 since there was no interaction between MT and p53. Our observations suggest that MT in its apo-form interacts with p53 in the cell and that this may prevent binding of p53 to DNA and at the same time may prevent degradation of apo-MT. Thus p53 may not be able to act as a transcriptional factor, and modulate gene transcription and apoptosis. These results also suggest that apo-MT/MT couple may be involved as a control mechanism to regulate p53 activity.

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