Reversible zinc exchange between metallothionein and the estrogen receptor zinc finger

Danielle F. Cano-Gauci**, Bibudhendra Sarkar*

Department of Biochemistry Research, The Hospital for Sick Children, 555 University Ave. Toronto, ON M5G 1X8, Canada
Department of Biochemistry, University of Toronto, Toronto, ON M5S 1A8, Canada

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Abstract We report here the first demonstration that reversible metal exchange occurs between metallothionein (MT) and full-length estrogen receptor (ER). Specific binding of ER to estrogen response element is inhibited in the presence of 40 μM thionein and restored by 120 μM zinc. Moreover, ER in metal-depleted nuclear extracts exhibits reduced DNA binding which can be restored by 140 μM native MT. Hence, thionein inhibits DNA binding by abstracting zinc from functional ER while native MT is capable of restoring binding to metal-depleted extracts by donating metal to ER. This indicates MT may be an important physiological regulator of intracellular zinc and/or other metals.

Key words: Metallothionein; Zinc; Zinc finger; Estrogen receptor

1. Introduction

Metallothioneins are small, 6 kDa proteins capable of binding 7 equivalents of bivalent metal ions. The metals form metal thiolate clusters through the high number of cysteine residues: 20 of 60 amino acids are cysteine. MTs are expressed in a tissue-specific manner, can be induced by a number of chemical and physical stresses including exposure to heavy metals, glucocorticoids and heat shock and are postulated to be involved in homeostasis of essential metals [1]. MTs may also be involved in the cellular response to oxidative stress [2,3]. For example, it appears that MT induction in the kidney reduces the renal toxity of cisplatin [4]. Zinc, one of the metals normally bound to MT, is an important constituent of many proteins and enzymes. We have focused our studies on the ER, a zinc-finger containing transcription factor. The ER is comprised of discrete domains constituting the ligand binding, transactivation, and DNA-binding domains. Within the DNA-binding domain, 8 cysteine residues coordinate 2 zinc atoms in a tetrahedral configuration, forming 2 non-equivalent zinc-finger-like motifs [5]. The DNA-binding domain also contains a dimerization signal: it is the homodimer which binds to the estrogen response element (ERE), a 15 base pair DNA sequence, 5′-AGGTCAnnnTGACCT [6].

The physiological source of the zinc in the ER remains unclear. One postulate is that MT may act to regulate the intracellular availability of zinc. In fact, MT which has been stripped of its zinc (henceforth called thionein) has been shown to abstract zinc from the zinc-containing transcription factors Sp1 [7] and TFIIIA [8], resulting in altered DNA binding and ultimately in altered gene regulation. Both Sp1 and TFIIIA are zinc finger-containing transcription factors of the 2Cys-2His type, as opposed to the ER which utilizes 4 cysteine residues to coordinate its zinc. The present study was carried out to determine whether we could observe metal exchange between the 4Cys ER zinc finger and MT. We report here that reversible metal exchange occurs between MT and the ER: thionein can extract zinc from the ER zinc finger and fully metal-bound MT restores zinc binding to metal-depleted ER. This is in contrast to what has been reported for Sp1: thionein extracts zinc from this transcription factor but MT does not donate the metal back to zinc-depleted SP1 [7].

2. Materials and methods

2.1. Proteins

The human breast carcinoma cell line MCF7, which overexpresses the ER, was obtained from ATCC. It was maintained in α-minimum essential medium (Gibco/BRL, Burlington ON, Canada) with added ribonucleosides and deoxyribonucleosides as well as penicillin, streptomycin and 10% fetal calf serum (Cansera, Mississauga, ON, Canada) in a humidified atmosphere of 5% CO_2. Nuclear extracts were made from confluent MCF7 cells as described [9] except that 25 μg each of the protease inhibitors leupeptin, aprotinin and antipain (Sigma-Aldrich, Missisauga, ON, Canada) were added with each sequential suspension in buffer. In addition, metal depleted nuclear extracts were prepared by the same procedure except that nuclei were homogenized in buffer in which the EDTA concentration was increased from 0.2 mM to 4 mM. In the final dialysis step, the EDTA concentration was 0.2 mM or, for the metal-free extracts, 4 mM.

Rabbit liver MT was obtained from Sigma-Aldrich, Canada. Apometallothionein, or thionein was prepared as described [10]. Briefly, 15 mg MT were dissolved in 1.5 ml 20 mM Tris-HCl, pH 8.6 in the presence of 1.5 mg DTT and incubated for 60 min at room temperature. The pH was rapidly adjusted to 1.0 using 1 M HCl. The sample was spun in an Eppendorf centrifuge and the supernatant was loaded onto a Sephadex G-50 column equilibrated with 10 mM HCl and eluted with the same solution, at 4°C. Metal-complexed MT exhibits a diagnostic absorbance at 254 nm; thionein at 220 nm. Therefore, the UV absorbance of all fractions was read at 20 nm (ε_{220}=48 200 M^{-1} cm^{-1}). The appropriate fractions were pooled and lyophilized after the pH was adjusted to 2.0–2.5. In some attempts where the ratio of A_{254}/A_{220} was relatively high, indicative of residual metal bound to the thionein, lyophilized protein was dissolved in 2 ml of 10 mM 1,10-phenanthroline, 10 mM HCl and 5 mM DTT and incubated at room temperature for 3 h, followed by dialysis (Spectrapor 6, MW cutoff 1000, obtained from Spectrum, Houston, TX) against 10 mM HCl and 5 mM DTT for 3 h at 4°C. Sample pH was adjusted to pH 2.5 before lyophilization. All solutions were either made with metal-free water which had been passsed over a Chelex 100 (BioRad, Mississauga, ON, Canada) column or were passed over the column themselves. Metal-free glassware, cleaned in 10% HCl overnight was used in all experiments requiring metal-free conditions.

*Corresponding author. Fax (1) (416) 813-5379.
E-mail: bsarkar@resunix.ri.sickkids.on.ca

***Present address:* Ontario Cancer Institute, 610 University Ave., Toronto ON M5G 2M9, Canada.

Abbreviations: ER, estrogen receptor; ERE, estrogen response element; MT, metallothionein

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2.2. Gel mobility shift assay

To assess the DNA-binding properties of ER in the nuclear extracts in the presence or absence of MT, thionein and exogenously added zinc, gel mobility shift assay was used. Generally, 20–25 μg nuclear extract protein (as determined by the Bradford assay [11]) were incubated with MT or thionein at room temperature in metal-free buffer containing 0.02 M HEPES, 0.1 M NaCl and 20% glycerol and 1 μl 5 mg/ml double-stranded poly(dI-dC) (Pharmacia, Baie D'Urfe, Quebec, Canada) to prevent non-specific DNA binding. Appropriate metals were added after 20 min. Metal and protein concentrations are indicated in the figure legends. Double stranded DNA comprising the ERE consensus sequence:

5′ GATCTAGGTCAGACTGACCTC
3′ ATCCAGTCTGACTGGAGAGCT

was end-labelled using [^{32}P]dATP and polynucleotide kinase and purified by separation on and elution from a 20% acrylamide gel. Radiolabelled DNA was added to the nuclear extract/metal/MT/thionein mixture and incubated for 20 min at room temperature before loading on a preequilibrated (at least 30 min) 5.6% polyacrylamide gel in 0.5×Tris-borate-EDTA. Gels were run at 100 mV, constant voltage for approx. 2 h, dried and autoradiographed, usually overnight.

3. Results

3.1. Specific binding of ER to ERE is inhibited by thionein

ER in nuclear extracts prepared from MCF7 cells binds to the ERE consensus sequence in a specific manner. Fig. 1, lane 1 shows 2 major DNA-binding complexes (corresponding to the homodimer, higher MW band, and the monomer, the lower MW band) which are retarded or 'shifted' in comparison to free DNA with no extracts added (lane 6). In the presence of excess non-radiolabelled ERE (lane 2), however, the binding is competed out, demonstrating the specificity of binding. This specific binding is also inhibited in the presence of 40 μM thionein (lane 3) but not in the presence of 100 μM MT alone (lane 5). The observed inhibition of DNA binding, therefore, is not due to non-specific protein effects (i.e. MT) but rather is due to extraction of zinc from ER by thionein. The addition of 120 μM zinc acetate to the nuclear extracts in the presence of thionein results in the restoration of binding of ER to its cognate DNA (lane 4). It appears, therefore, that thionein is able to abstract zinc from the ER present in the MCF7 cell nuclear extracts. The loss of zinc results in reduced binding to DNA and is reversible upon reintroduction of zinc in the form of zinc acetate. The next set of experiments was designed to determine if reintroduction of zinc in another form, i.e. native MT, could also restore binding of nuclear extracts to the ERE.

3.2. Reconstitution of binding in the presence of MT or zinc

Fig. 2, lane 1, shows that binding of ER to labelled DNA is significantly reduced in nuclear extracts prepared in the presence of 4 mM rather than 0.2 mM EDTA. Although the ER in these 'metal-free' extracts exhibits a low level of binding to ERE, the binding is increased in a dose-dependent manner in the presence of 0.7 (lane 2) and 1.4 mM zinc acetate (lane 3). Addition of unlabelled ERE (lane 4), indicates the specificity of binding (ER/ERE complex is indicated by an arrow). As well as being reconstituted in the presence of zinc, we report the significant observation that MT is also capable of restoring DNA binding of metal-depleted ER. Binding to ERE is restored in the presence of 140 μM MT (lane 5) and to a greater extent by 420 μM MT (lane 6). In fact, metal-depleted ER binding to ERE could be restored by both zinc and MT at

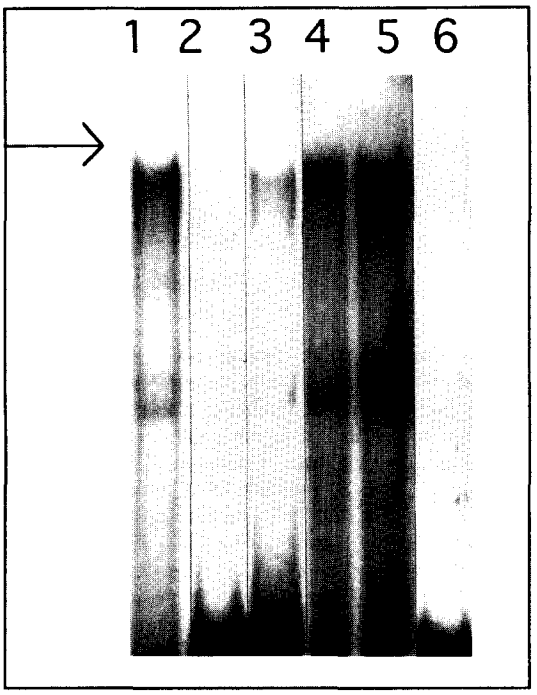

Fig. 1. Specific binding of ER in MCF7 nuclear extracts to ERE is inhibited by thionein. 25 μg nuclear extract protein were incubated with end-labelled ERE as described in section 2, with the following additions: (lanes) 1, no additions; 2, 1 μg unlabelled ERE; 3, 40 μM thionein; 4, 120 μM zinc acetate; 5, 100 μM Cd/Zn MT; 6, only free, labelled DNA, no nuclear extracts added. ER/ERE complex is indicated by the arrow.

concentrations as low as 150 and 100 μM, respectively (data not shown). It seems that MT donates its metal to metal-dependent ER allowing it to bind to DNA. This reconstitution is similar to that which occurs when metal alone is added to metal-depleted ER. Additional MSA experiments were carried out in the presence of either MT or MT which has been previously Chelex-treated. We expect such treatment to remove zinc loosely associated with the commercial MT used in these experiments. Both Chelex-treated and untreated MT were able to restore DNA binding of metal-depleted nuclear extracts, indicating that the reconstitution is due to zinc that is bound to MT via thiolate clusters and not non-specificially bound zinc (data not shown).

Therefore, in addition to the ability of thionein to remove zinc from ER zinc fingers, thus inhibiting DNA binding, as seen in Fig. 1, native MT is able to restore DNA binding to metal-depleted ER-containing nuclear extracts by donating metal back to the ER zinc finger. Hence, the transfer of metal between MT/thionein and ER appears to be a reversible reaction. It is important to note that when either MT or zinc acetate were added to fully metal-bound nuclear extracts, there was no enhancement of DNA binding above control levels at concentrations of up to 7 mM zinc acetate or 700 μM MT, respectively (data not shown).

4. Discussion

The results presented here indicate that reversible metal exchange occurs between MT and/or thionein and the ER. Specifically, we have shown that ER in nuclear extracts prepared from the human breast cancer cell line MCF7, bind in a

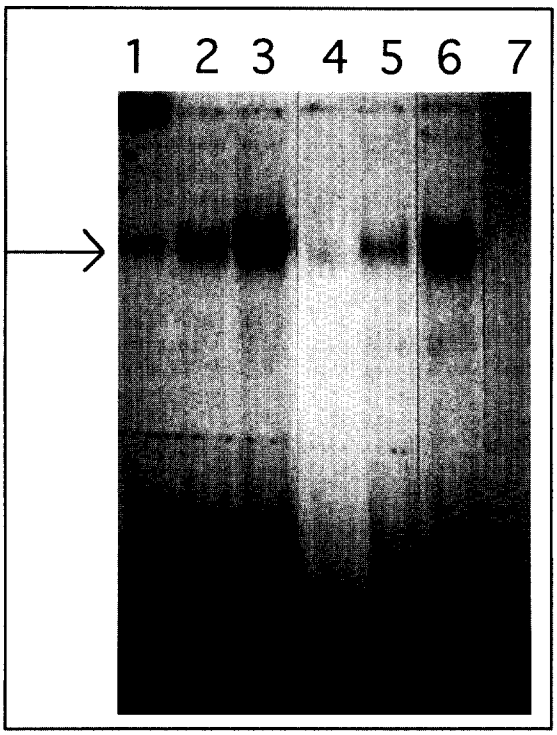

Fig. 2. Restoration of DNA binding of ER in metal-depleted nuclear extracts by both zinc and native MT. 20 μg of metal-depleted nuclear extracts were incubated as described in section 2 with the following additions: (lanes) 1, no additions; 2, 0.7 mM zinc acetate; 3, 1.4 mM zinc acetate; 4, 1 μg unlabelled ERE; 5, 140 μM native MT; 6, 420 μM MT; 7, free DNA, no nuclear extracts added. ER/ERE complex is indicated by the arrow.

specific manner to the consensus ERE. This binding is inhibited in the presence of 40 μM thionein, which apparently acts to abstract the zinc from the ER zinc fingers. Addition of 120 μM zinc acetate restored the binding to ERE. Moreover, ER from metal-depleted nuclear extracts exhibited reduced DNA binding. Binding of metal-depeted ER to DNA was restored by the addition of either 700 μM zinc acetate or 140 μM native MT. Binding increased in a dose-dependent manner in the presence of either zinc acetate or MT. Hence, it appears that the metal exchange between MT/thioneins and ER is reversible. That is, thionein is capable of abstracting zinc from functional (with respect to DNA binding) ER and native MT is capable of restoring DNA binding of ER in metal-depleted extracts. This effect is similar to that of addition of non-protein bound zinc in the form of zinc acetate. We previously demonstrated that metal replacement within the ER was possible. For example Cd and Co [12], as well as Fe [13] were able to restore DNA binding to zinc-depleted ER. It is important to note that our previous studies with the metal-substituted ER zinc finger were carried out using the ER DNA-binding domain only, while the present study utilized the full-length ER present in MCF7 cell nuclear extracts. It appears therefore, that the presence of the other ER domains, i.e. the ligand binding and transactivation domains do not interfere in the metal exchange we observed.

The ability of thionein to extract zinc from the 4Cys zinc finger configuration of the ER is consistent with reports that indicate similar zinc loss from 2Cys-2His TFIIIA and Sp1 zinc fingers. These studies reported that approx. 10–20-fold less thionein than was used in our study, i.e. 4 and 1.7 μM could inhibit DNA binding by Sp1 [7] and TFIIIA [8], respectively. The differing ability of thionein to extract zinc from 4Cys versus 2Cys-2His zinc fingers may reflect the structural differences which could account for different affinities for zinc. In fact, the zinc-binding motif of steroid hormone receptors has been called a zinc 'twist' rather than a classical zinc 'finger' of the TFIIIA or Sp1 type to recognize these structural differences [14]. Nonetheless, the observed reversibility of zinc exchange in this study suggests that ER zinc fingers have an affinity for zinc similar to that of MT. While a recent study demonstrated metal exchange between a single synthetic Sp1 zinc finger peptide and the alpha domain of human MT [15], a contrasting report, [8] indicated MT does not donate zinc to the zinc-depleted Sp1 transcription factor. In fact, a range of K_d values for zinc in zinc fingers (10^{-12} M [16] and both high and low affinity, i.e. 10^{-8} and 2.6×10^{-5} M [17] for TFIIIA; 2×10^{-12} M for a consensus, synthetic finger [18]) as well as in MT (5×10^{-13} M [19] and 6×10^{-10} M [15]) have been reported. These discrepancies most likely reflect the variety of experimental techniques used, including gel mobility shifts, equilibrium binding, peptides versus full-length proteins etc. Further studies are needed to clarify the basis of these differences. Alternatively, the difference in thionein's ability to inhibit DNA binding may reflect the high level of ER which is overexpressed in MCF7 cells, as compared to the level of Sp1 in HeLa cells or TFIIIA in frog oocytes.

The reversible zinc exchange we report here indicates that MT may be an important physiological regulator of zinc equilibria between cell storage sites, free cytosolic zinc and other proteins in which zinc has a structural and/or functional role. The homeostasis of other metals may also be under the influence of MT. MT can undergo metal exchange reactions [20] and binds many other metals, including iron [21,22]. Its ability to bind iron is especially interesting in light of the fact that Fe-substituted zinc finger generates free radicals which cleave DNA [13]. This raises the possibility that MT may act to protect against oxidative stress by binding excess iron. The physiologival relevance of $^{\bullet}OH$ radical induced DNA modifications is an important one since $^{\bullet}OH$ radicals have been reported in many cancerous human tissues [23,24]. A recent report [25] indicated that MT protects DNA from copper but not iron-induced cleavage in vitro. However, that study was carried out using free metal salts, i.e. not protein-bound, and non-specific calf thymus DNA. Another study indicated that human αMT has a higher affinity for nickel than zinc, possibly preventing nickel-induced toxicity [15]. In our study, we observed reversible metal exchange between MT and ER, two full-length proteins, as opposed to peptides, using these proteins as the metal donor and acceptor in the exchange reaction. Therefore, we should be able to utilize this system to investigate the potential role of MT both as a physiological regulator of intracellular metal equilibria and as a protector against oxidative stress induced by iron or other metals.

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