Purification of mouse MEP-1, a nuclear protein which binds to the metal regulatory elements of genes encoding metallothionein

Simon Labbé, Lucie Larouche, Donald Mailhot and Carl Séguin*
Centre de Recherche en Cancérologie de l'Université Laval, L'Hôpital-Dieu de Québec, 11 côte du Palais, Québec G1R 2J6 and Département de Physiologie, Faculté de Médecine, Université Laval, Québec G1K 7P4, Canada

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ABSTRACT
Metal regulatory elements (MREs) shared by metallothionein (MT) gene promoters are essential for metal induction of MT genes. MEP-1, a nuclear protein which binds to these elements has been purified from heavy metal-resistant mouse L cells using footprinting, Southwestern and UV cross-linking techniques to assay its binding activity. The purification scheme, starting from crude nuclear extracts, involved a combination of heparin-Sepharose and MRE-DNA affinity chromatography. The purified protein preparation showed a single polypeptide band of 108 kDa on polyacrylamide gel electrophoresis, and 2D-gel analyses revealed the presence of a protein species migrating as a single population of approximately 110 kDa. MEP-1 does not appear to be glycosylated since it eluted with the flow-through on a Wheat Germ Sepharose column. It was retained by a zinc-Chelating Sepharose column suggesting that amino acid residues (i.e., cysteine, histidine) which have an affinity for zinc ions are exposed on the protein surface. Binding studies with the purified protein indicated that it binds specifically to MRE sequences and that the binding can be abolished by a point mutation in the MRE core consensus sequence or by the addition of the chelating agent 1,10-phenanthroline. Binding activity can be restored by the addition of zinc ions to the chelated protein. These results suggest that MEP-1 is one of the major proteins interacting with MRE sequences.

INTRODUCTION
The homeostatic status of the organism is maintained through numerous mechanisms interacting with each other and influencing the selective activation of particular sets of genes. Many agents present in the environment can have a profound deleterious effect on intracellular homeostasis and affect gene expression. One class of such environmental agents are the heavy metals such as cadmium, zinc and copper. The best known proteins to bind these metal ions are the metallothioneins (MTs), a class of small cysteine-rich proteins present in many different tissues and cell types from yeast to human (reviewed in 1,2). MT gene transcription can be induced by metal ions and by various stresses and hormones but metals are the primary inducers. The precise mechanisms by which the cell senses the metal concentration and transduces the information to the gene are not known. It is believed that one or more intracellular proteins could act as limiting high affinity-factors for metal-dependent MT gene activation (3).

Metal activation of MT gene transcription is dependent on the presence of short cis-acting elements termed MREs (metal regulatory elements) present in six imperfect copies (MREa through MREf) in the 5' flanking region of the mouse MT-I gene (4—7) and involves trans-acting factor(s) interacting with the MREs as revealed by in vitro (8—18) and in vivo (19,20) binding studies. Of particular interest with respect to MRE-binding proteins is the nuclear protein(s) interacting with the MREd element of the mouse MT-I gene. Several lines of evidence support the idea that the MREd-binding protein(s) is important for metal-inducible transcription. First, MREd is the strongest element in vivo (7). Second, DNA-binding activity of the protein(s) interacting with the MREd element is inactivated by the chelating agents EDTA and 1,10-phenanthroline in vitro, and can be restored by zinc ions (15). Third, nuclear extracts prepared from heavy metal-resistant L cells (L50) contained approximately four times more MREd-binding activity than extracts prepared from induced L cells (C. Séguin, unpublished data).

Using protein blotting and UV cross-linking procedures, we showed that MEP-1, a mouse nuclear protein of 108 kDa, binds with high affinity to the MREd element (9,14). In addition, MEP-1 binds to the other MRE sequences present in the promoter of the mouse MT-I gene with affinities that are proportional to their relative transcriptional strength in vivo. MEP-1 also binds to MREs of the human MT-IIa and trout MT-B genes and a protein of similar size is present in HeLa cells.

To investigate the role of MEP-1 in the complex formed on MRE elements as detected by footprinting, we purified the protein...
to homogeneity and characterized its binding properties. Footprinting studies demonstrated that purified MEP-1 specifically binds to MRE sequences and that it is sufficient to produce a specific footprint on the mouse MREd element. MEP-1 binding activity is also inhibited by the chelating agent 1,10-phenanthroline and can be restored by zinc ions.

MATERIALS AND METHODS

Cell culture and extract preparation

Heavy metal-resistant mouse L50 cells (obtained from D.H. Hamer) were grown in suspension in SMEM in the continuous presence of 15 μM CdCl₂ (15). Crude nuclear extracts were prepared as described (8), except that the final dialysis step was omitted. Buffers contained 5 μM CdCl₂ and 50 μM ZnCl₂.

Binding assays

ExonucleaseIII (ExoIII) footprinting analyses were performed as described by Séguin and Hamer (8) with the exception that poly (di-dC)₂ (Pharmacia) was used instead of φX174 DNA as non specific competitor DNA; 1 μg was included in the reaction mixtures to assay crude nuclear extracts or heparin-Sepharose fractions and 20 to 50 ng for reactions with the purified MEP-1 fractions. The MT fragment used as the probe was 5’ end-labelled at position +64 and extends to position −200 relative to the start point of transcription. Gel-purified oligodeoxynucleotide (oligomer) competitors were annealed to form double-stranded DNA. The concentration of each single stranded oligomer DNA was measured by spectrophotometry. The percentage of double stranded molecules was evaluated by gel electrophoresis and was always greater than 95%. Competitors were added together with the probe, and binding was allowed to proceed for 20 min at 24°C. The MRE oligomers mMREds (14) and MUT-5 (15) used in competition experiments have been described.

DNasel footprinting assays were performed as described (21) on a mouse MT fragment 5’ end-labelled at position −41 and extending to position −200 relative to the start point of transcription.

The protein blotting (Southwestern; 9) and the UV cross-linking (14) assays were performed with double stranded oligomers. Typically, 10–60 μg of crude nuclear extract or of the different chromatographic fractions were used in each reaction.

Protein chromatography

Twelve ml of L50-cell crude nuclear extracts (10 μg total proteins/μl) were applied onto a 2-ml heparin-Sepharose CL 6B (Pharmacia) column equilibrated with buffer III (8) and eluted with a NaCl step gradient (325–1500 mM) in buffer III. Fractions containing the DNA-binding activity were pooled, diluted to a final concentration of 130 mM NaCl with buffer III (without NaCl) and loaded onto an 0.5-ml DNA-affinity column containing the concatenated oligomer mMREa (14), according to the method of Chodosh et al. (22). The bound proteins were eluted with a step NaCl gradient (130–1000 mM). The active fractions were pooled, diluted with buffer III to 130 mM NaCl and rechromatographed on the same affinity column, resulting in purified MEP-1. Throughout purification, all buffers were supplemented with 5 μM CdCl₂ and 50 μM ZnCl₂. BSA (250 μg/ml; Gibco/BRL) was also added to the elution buffers for the heparin-Sepharose column and the first round of affinity chromatography.

RESULTS

Purification of MEP-1

A factor(s) present in L- (8, 15) and L50- (9, 14; Fig. 2A, lane 3) cell crude nuclear extracts bound to the MREd region of the mouse MT-I gene in a zinc dependent manner (15). The binding site maps over MREd together with the distal portion of MREc (nucleotides −153 to −127) (8). Using an MREd oligomer probe, a 108 kDa molecular weight protein, termed MEP-1, was detected in crude nuclear extracts by Southwestern analyses (9,14; Fig 1B, lane 1). In UV cross-linking experiments, MEP-1 forms a complex migrating on a denaturing gel with an apparent Mr of 115 000 (14; Fig 2B, lane 1).

The purification of MEP-1 was followed using SDS-PAGE, Southwestern and UV cross-linking assays. Heavy metal-resistant

![Figure 1. Purification of MEP-1.](image-url)
Table 1. Purification of mouse MEP-1

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total protein (mg)</th>
<th>Total activity (Unit)</th>
<th>Specific activity (Unit/µg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<tr>
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<td>20</td>
</tr>
<tr>
<td>DNA affinity 2</td>
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<td>25,000</td>
<td>25,000</td>
<td>6,800</td>
<td>6</td>
</tr>
</tbody>
</table>

a Results obtained in a typical experiment, starting with a crude nuclear extract prepared from 6 l of L50 cells (5×10⁵ cells/mL).
b One thousand units of activity is defined as the amount of protein required to obtain the signal produced by the quantity of MEP-1 present in 270 µg (30 µl) of a standard crude nuclear extract, under the conditions of our Southwestern assay, after a 1 hr exposure at -80°C, using 1×10⁶ cpm/ml of a mouse MREa oligonucleotide probe freshly labelled at 1500 cpm/µmol.

Figure 2. Binding assays of representative chromatographic fractions. (A) ExoIII footprinting analysis performed in the presence of 1 µg (lanes 2–5) or 20 ng (lanes 6–9) of poly (dI-dC). Lanes: 1, probe alone without extract or digestion with ExoIII; 2, probe digested with ExoIII in absence of extract; 3, crude nuclear extract; 4 and 5, heparin-Sepharose fractions; 6 and 7, first DNA affinity; 8 and 9, second DNA affinity; 4, 6 and 8, flow through; 5, 7 and 9, 650 mM salt fractions. The arrows indicate the ExoIII stop at the -153 boundary in the MREd region. The binding of purified MEP-1 upstream the MREd region (lanes 7, 9) is non specific since it can be competed out by raising the amount of poly (dI-dC) to 50 µg (see Fig. 6). (B) UV cross-linking assay. Lanes: 1, crude nuclear extract; 2 and 3, heparin-Sepharose column; 4 and 5, second affinity column; 2 and 4, flow through; 3 and 5, 650 mM NaCl fractions. Samples in lanes 1 to 3 were run on one gel and those in lanes 4, 5 were run on a different gel. The arrow indicates the 115 kDa DNA-protein complex.

Figure 3. Two dimension gel analysis performed with 30 ng of purified MEP-1 (MEP-1; aliquot of the 650 mM fractions from the second DNA affinity column) or an identical volume of sample buffer (Buffer). Numbers on the left refer to molecular weight markers (Da) and were determined by running a set of protein markers (BioRad). The arrow designates MEP-1.

L50-cell nuclear extracts were chosen to purify MEP-1 because the MREd-binding activity is enhanced in these cells compared to wild type L cells. In heparin-Sepharose chromatography, MEP-1 binding activity was eluted in the 650 mM salt fractions. We observed that dialysing the active fractions against a low salt buffer led to an important loss of activity due, possibly, to aggregation or sticking of the proteins on the dialysis membrane. Therefore, we diluted the active fractions to allow binding on the affinity matrix. Figure 1 shows the overall activity and protein profile of MEP-1 purification. The enrichment of MEP-1 activity was estimated about 7000-fold (Table 1). The purified protein preparation shows a single polypeptide band of 108 kDa on polyacrylamide gel electrophoresis (Fig. 1A, lanes 20–22), and 2D-gel analyses revealed the presence of a protein species migrating as a single population of approximately 110 kDa (Fig. 3).

Chelating Sepharose charged with a suitable metal will selectively retain proteins when amino acid residues (i.e., cysteine, histidine) which have an affinity for the chelated metal ions are exposed on the protein surface (25). MEP-1 is retained by a Chelating Sepharose matrix charged with zinc while most of the protein was found in the flow-through (data not shown). MEP-1 does not appear also to be glycosylated since it eluted with the flow through on a Wheat Germ Sepharose column (Fig. 4) contrary to transcription factor Sp1 which was retained on the column, as assayed by gel retardation analyses (data not shown). Some trans-acting regulatory proteins such as Sp1 (26) and HNF1 (27) are glycosylated post-translationally and Wheat Germ Sepharose, a lectin affinity matrix which recognizes N-acetyl-D-glucosamine, is used for the purification of RNA polymerase II transcription factors (28).
DNA binding activity of purified MEP-1

Only the 108 kDa MEP-1 band was detected by the MREd probe in Southwestern and UV cross-linking assays (Figs. 1B and 2B). The footprint over MRE elements of affinity-purified MEP-1 is identical to that produced by factors present in crude extracts (Figs. 2A and 5A). In DNaseI footprinting analyses, MEP-1 bound to all three MREs present on the probe, namely MREc, MREd and MRee (Fig. 5A). DNaseI footprinting reactions performed with low amounts of purified protein showed that MEP-1 binds preferentially to the strong MREd element (Fig. 5B), thus indicating that MEP-1 has a higher affinity for MREd than for MREc and MRee. In order to assess the binding specificity of purified MEP-1, competition experiments were performed by ExoIII footprinting analyses. Formation of the complex at the level of MREd was inhibited by incubation with excess of the specific MREd oligomer but not by the mutated MUT-5 competitor (Fig. 6A), indicating that the complex is attributable to sequence-specific interactions. The mutation present in MUT-5 has been shown to completely abolish MT transcriptional activity in vivo (29). Purified MEP-1 also bound to the MREa and MReb elements of the trout MT-B gene (Labbé, S., Imbert, J., and Séguin, C., unpublished data). As shown in Fig. 6B, 1,10-phenanthroline abolished MEP-1 MREd-binding activity, but it could be restored by subsequent addition of ZnCl2. No other cations tested restored activity to the chelated protein. Therefore, MEP-1 requires zinc ions for its specific interaction with DNA.

DISCUSSION

We have purified the MRE-binding protein, MEP-1, from the heavy-metal resistant mouse L50 cells and have characterized the binding activity of the purified protein. Purified MEP-1 binds specifically to MRE sequences and produces a footprint identical to that produced by the factor(s) present in crude nuclear extracts. As for the MREd-binding activity present in crude extracts, MEP-1 requires zinc to bind the mouse MREd element, and a single nucleotide substitution introduced in the core MRE region...
at a nucleotide required for in vivo transcriptional activity of MREd (29) completely abolished MEP-1 binding activity as assayed by competition experiments.

In vivo competition experiments showed that increased MT gene transcription in response to metal induction is achieved by a positively acting transcription factor present in limited amount in monkey cells (30). It has been suggested that this factor would become activated by interacting with metal ions. While factors regulating metal responsive gene expression have been cloned and characterized in prokaryotes (31) and in yeast (32-35), such a factor has yet to be identified and isolated in vertebrates. MRE-binding proteins have been proposed to play a central role in MT gene transcription activation as metal regulatory transcription factors (3,36). We have previously shown that a nuclear factor requires zinc to bind the mouse MREd element and that the nucleotides recognized by this factor are the same as those required for in vivo transcriptional activity of MREd. This suggested a major role for the MRE-binding protein(s) in metal dependent activation of MT gene transcription. We show here that purified MEP-1 is sufficient to generate the footprint observed in vivo and can completely abolish MEP-1 binding activity as assayed by competition experiments.

The purification of MEP-1 provides an essential tool to answer the following questions. First, MEP-1 may play a central role in metal-dependent activation of MT gene transcription and have a zinc finger-like DNA binding domain and a transcription domain activated by zinc and other heavy metals; however, the biochemical effects such as a transcription domain could not be detected by the binding assays used in this study. Second, MEP-1 may belong to a family of MRE-binding regulatory proteins, each of which is specific for a single class of metal ions. Third, MEP-1 may be a general DNA-binding protein not central for metal regulation and, as for many DNA-binding proteins, it may have a specific requirement for zinc to bind DNA.

Recently, a HeLa-cell nuclear factor with a molecular mass of 112 kDa, MREBP, that recognizes MREs of the human MT-IIA gene was partially purified (17). Mobility shift assays and DNase I footprinting experiments indicate that MREBP binds specifically to several MREs present upstream of the human MT-IIA gene. Curiously, this protein also gave a strong footprint on MREG, an MRE-like sequence which contains two mismatches in the MRE core region (17) at positions essential for MEP-1 DNA-binding in vitro (14) and metal-induction of MT gene transcription in vivo (29). Other MRE-binding proteins have also been detected by different DNA binding assays. ZAP (12) and p39 (13) have been identified in rat cells, whereas MTF-1 (10), MRE-BF1 and MRE-BF2 (18) have been found in human cells. While the molecular weight of ZAP and of MTF-1 is not known, p39 is a 39 kDa protein, MRE-BF1, which binds to MREs in control cells, has an apparent molecular mass of approximately 86 kDa, and MRE-BF2 consists of two molecules of approximately 28 kDa and binds to MREs in metal-treated cells. The relationship between these proteins and MEP-1 is unclear and the cloning of the corresponding cDNAs will be required to establish the precise relationship among them.

While a direct assessment of the capacity of MEP-1 to bind metal ions has not been performed, we have shown that MEP-1 is retained on a zinc-Chelating Sepharose column, thus suggesting that this MRE-binding protein is able to bind zinc ions. In addition, it has been recently shown that a discrete fraction of rat liver nuclear proteins, representing native molecular weight ranges of approximately 20-2000 kDa and being eluted in the range of 300-1000 mM salt from an heparin-Sepharose column, binds both 65Zn and a trout MRE oligomer probe (37). More studies will be required to establish the precise relation between MEP-1, and this zinc-binding protein(s).

The assessment of MEP-1 as a metal regulatory protein and of the possible interactions with other MRE-binding proteins will require a specific metal dependent in vitro transcription assay. The purification of MEP-1 provides an essential tool to answer these questions.

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REFERENCES