Epitope-Tagged Metallothionein: Its Biochemical and Immunocytochemical Applications

Masami Ishido* and Junko Sayato-Suzuki

Division of Environmental Health Sciences National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba 305–8506, Japan

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We developed epitope-tagged metallothionein IIA cDNA by the addition of an 8-amino acid sequence, DYKDDDDK, at the N-terminus metallothionein using polymerase chain reaction. The fusion proteins were expressed by transfection in human embryonic kidney 293 (HEK-293) cells and were specifically recognized by commercially available antibody, an anti-Flag M2 monoclonal antibody. Furthermore, the fusion protein was detectable on a Western blot, whose migration was expectedly at 7000–8000 in an SDS-denatured form. Thus, availability of the epitope-tagged metallothionein will facilitate the investigation of cellular mechanisms of the protein such as its nuclear localization mechanism.

Key words — epitope-tag, metallothionein, cellular localization, Western botting

INTRODUCTION

Metallothioneins, a metalloprotein of low molecular weight (6000–7000), are cysteine rich (30%) intracellular proteins with high affinity for certain metals.¹⁻³⁾ High levels of an endogenous metallothionein bound to zinc and copper have been detected in mammalian liver during gestation and early postnatal period in various species, suggesting a biological role for metallothionein in metal homeostasis.¹⁻³⁾ Metallothionein is shown to sequester large amounts of toxic metals such as cadmium intracellularly and therefore it may play a significant role in metal detoxification.^{1–3)} It is also demonstrated that metallothionein can scavenge free radicals and may participate in a cellular antioxidant defense system.^{4,5)}

Although metallothionein was originally considered to be largely a cytoplasmic protein, there is evidence that metallothioneins are found localized in the cell nucleus under certain physiological circumstances.^{6–8)} Since metallothionein dose not possess a classical nuclear localization signal,^{9,10)} the molecular basis of nuclear localization of the protein remains unclear.

Therefore, as the first step for identifying the nuclear localization signals by the method of sitedirected mutagenesis of metallothionein IIA, we developed an epitope-tagged metallothionein IIA to trace the cellular localization of the recombinant protein *via* Flag-recognizable antibody.

MATERIALS AND METHODS

Synthesis of the Epitope-Tagged Metallothionein - An 8-amino acid epitope-tag sequence was inserted at the N-terminus of metallothionein IIA using a polymerase chain reaction (PCR) strategy.^{11,12} The oligonucleotide 5'-TAGCAAGCTTCCATG-GACTACAAGGACGACGATGACAAGGATCCC-AACTGCTCC-3' was used as the sense primer. This oligonucleotide has a Hind III restriction site followed by an initiation methionine codon (ATG), 24 bases coding for the DYKDDDDK epitope-tag sequence, and 15 bases of metallothionein sequence starting at codon 2. The anti-sense oligonucleotide primer was 5'-TAGCCTCGAGTCAGGCGCA-GCAGCTGCACTTGTCCGA-3'. This oligonucleotide has an XhoI cloning site, the stop site for translation, and 24 codons of metallothionein C-terminal coding sequence. The PCR reaction mixture contained human metallothionein IIA cDNA pSV as a template DNA, 0.25 mM dNTP, 100 pmol specific primers, and *pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.). Thirty-five cycles of PCR were carried out in a Thermal Controller (Perkin-Elmer; model PJ 2000) set to 94°C for 1 min, to 50°C for 1 min, and to 72°C for 3 min, ending with 5 min at 72°C. The PCR products were cloned into the HindIII and XhoI sites of pcDNAI (Invitrogen, Carlsbad, CA, U.S.A.) and were verified by sequencing.

Sequencing — The presence of the epitope sequence was confirmed by sequencing using an Auto

^{*}To whom correspondence should be addressed: Division of Environmental Health Sciences National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba 305–8506, Japan. Tel.: +81-29-850-2396; Fax: +81-29-850-2880; E-mail: ishidou@ nies.go.jp

Read sequence kit (Amersham Biosciences, NJ, U.S.A.).

Cell Culture and Transfection — Human embryonic kidney 293 (HEK-293) cells (RCB1637; Riken, Tsukuba, Japan) were grown in Dulbecco's modified Eagle's Medium (Sigma Chemical Co., St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD, U.S.A.), 4.5 mg/ml glucose, penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a humidified atmosphere of 95% air, 5% CO₂ at 37°C. The cells were subcultured (1 : 4) 2 to 3 times per week. Cell viability was determined by trypan blue exclusion method.

The tagged human metallothionein IIA plasmid (2 μ g) was transfected into the HEK-293 cells, plated at 2.6 × 10⁴ cells/cm² 16 hr before transfection, using LipofectAMINE PlusTM (Life Technologies, Inc.) according to the manufacturer's instructions.

Immunocytochemistry — Immunostaining was carried out as described previously.^{13,14)} Cells were fixed in 4% paraformaldehyde, washed twice with phosphate- buffered saline (PBS), and permeabilized in 0.5% Triton X-100 for 5 min on ice. The section was then blocked with 10% normal horse serum plus 4% bovine serum albumin for 30 min at 4°C. The sample was incubated with primary monoclonal anti-Flag M2 antibody (Eastman Kodak Company, CT, U.S.A.) in the presence of 4% bovine serum albumin and 0.05% Triton X-100. After three washes with PBS containing 0.1% Tween 20, the sample was incubated with FITC-labeled secondary goat anti-mouse IgG (Sigma) for 60 min at room temperature.

Following labeling, the cells were washed with PBS twice. The cover slips were mounted in 50% glycerol solution onto a slide and then directly surveyed under a fluorescence microscope (E400; Nikon, Tokyo, Japan). Images were captured using FlashPoint camera software.

Cell Fractionation and Western Blot Analysis —— The fractionation of the cell lysates and Western blotting were performed as described previously.^{15–17)} Transfected cells were collected by centrifugation at $1000 \times g$ for 5 min and homogenized in 20 mM Hepes, pH 7.8, containing 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride (Wako Co., Osaka, Japan), 5 µg/ml pepstatin (Peptide Institute Inc., Osaka, Japan), and 5 µg/ml leupeptin (Peptide Institute Inc.). The homogenates were kept in ice for 10 min. After centrifugation at 2000 rpm for 10 min, the supernatants were used as a cytosolic fraction. Protein concentrations were measured with a bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL, U.S.A.) using bovine serum albumin as a standard. Twenty micrograms of proteins were subjected to 15% polyacrylamide gels containing 0.1% SDS under reducing conditions.¹⁸⁾ Proteins in an SDS gel were electrophoretically transferred at 2 mA/cm² for 20 min onto Immobilon membrane (Millipore Co., Osaka, Japan) in 25 mM Tris, 192 mM glycine, and 20% methanol with an Atto semidry horizontal electrophoretic transfer unit (Atto, Tokyo, Japan). The membrane was blocked with 8% casein in PBS containing 0.1% Tween 20 at room temperature for 3 hr. The transferred membrane was then incubated with monoclonal antibodies against Flag M2 (1 μ g/ml; Eastman Kodak Company) in PBS containing 0.1% Tween 20 and 4% bovine serum albumin overnight at 4°C. After incubation with primary antibodies, the sheets were washed three times for 5 min each time with PBS containing 0.1% Tween 20, and the antibodies were detected with horseradish peroxidase-conjugated secondary IgG using an enhanced chemiluminescence (ECL) Western blotting detection kit according to the instructions of the manufacturer (Amersham Biosciences). Gels were calibrated with prestained molecular markers (Bio-Rad, Hercules, CA, U.S.A.).

RESULTS AND DISCUSSION

Recombinant metallothionein IIA was modified by addition of an 8-aa sequence,DYKDDDDK. We inserted the DYKDDDDK epitope-tag sequence at the N-terminus of the metallothionein beginning behind the initiation methionine (Fig. 1). This 8-aa sequence is recognized by the anti-Flag M2 antibody. The vector contains a cytomegalovirus promotor (CMV), splice segment and polyadenylation signal. The fusion proteins were controlled under the CMV promotor. The partial sequence of the construct around the initiation confirmed that the insertion of epitope-tag sequence was in-frame of metallothionein IIA cDNA (data not shown).

To verify whether the Flag-metallothionein construct expresses the correct fusion protein, the synthesis of the fusion protein was characterized in HEK-293 cells by transfection. The control experi-



Fig. 1. Position of the 8-aa Epitope Tag in the Metallothionein IIA cDNA

A schematic map of epitope tag constructs is shown.





HEK-293 cells were transfected with mock (A) or 2 μ g Flagmetallothionein IIA cDNA (B and C). After 3 days, the cells were fixed, followed by staining with no primary antibody (B) or with monoclonal anti-Flag M2 antibody (A and C). All cells were then incubated with FITC-labeled secondary antibody. Scale bar = 50 μ m.

ments with mock (Fig. 2A) or with no primary antibody (Fig. 2B) demonstrated no fluorescent stain on the culture. A majority of stain of the fusion protein was in the cytoplasm (Fig. 2C)

To examine the availability of anti-Flag M2 antibody for Western blot analyses, the cell lysates expressing the fusion proteins in HEK-293 cells were subjected to 15% SDS-polyacrylamide gel electrophoresis in the presence of 40 mM dithiothreitol, followed by Western blotting. As shown in Fig. 3, there was no immunoreactivity with mock-transfected cell lysates (lane 1). The fusion proteins were specifically detected by anti-Flag M2 antibody at



Fig. 3. Western Blot Analysis of Flag-Metallothionein Proteins HEK-293 cells were transfected with mock (lane 1) and the epitope-tagged metallothionein (lane 2), and then the cell lysates were homogenized. After fractionation, the supernatants (20 μ g of proteins) were applied to a 15% SDS-polyacrylamide gel electrophoresis in the presence of 40 mM dithiothreitol, followed by Western blotting with anti-Flag M2 antibody. The positions of size markers (kDa) are shown on the left margin.

an expected position (7-8 kDa; lane 2).

In this study, we developed the epitope-tagged metallothionein and found that the epitope-tag encoded in cDNA used for gene transfer studies provides an easy detection method specific for the recombinant protein, which is recognized specifically by the anti-Flag M2 antibody. The anticipated application of the Flag-metallothionein IIA transfection system is that it will allow for sequence analysis of the functional role of the metallothionein IIA sequence at various regions and amino acid residues. Mutational analysis of the Flag-tagged metallothioneins opens several potential avenues for investigations of the nuclear localization mechanism of the protein.

Recent discovery of the apoptogenic nature of cadmium has revealed that metallothionein would be involved in a necrotic phase for the metal detoxification,^{16,19-23} whereas a putative oncogene such as Bcl-2 but not c-MYC largely attenuated cadmium toxicity in an apoptotic phase.^{16,24} For further toxicological study of metallothionein using the Flagmetallothionein, a stable clone must be established since the population of the expressed fusion proteins is low in a transient expression system (Fig. 2C). This is under investigation in our laboratory.

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