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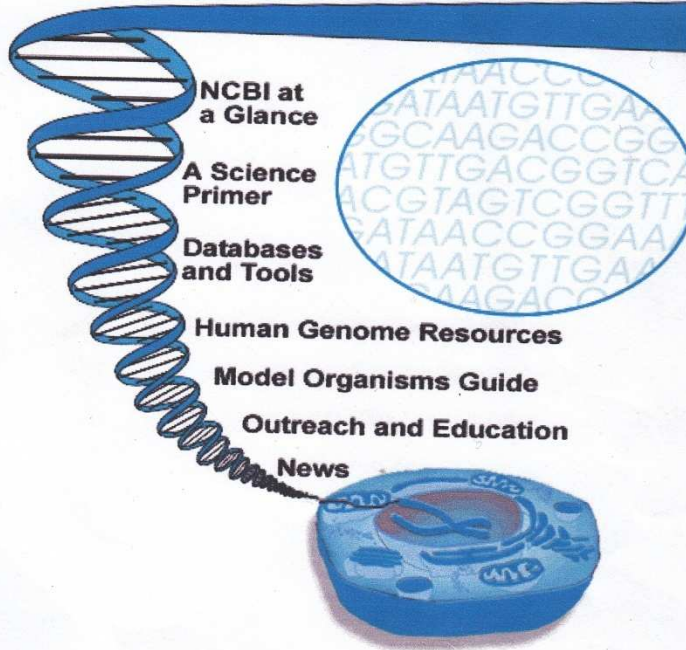
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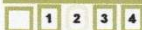
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The laminin binding protein p40 is involved in inducing limb abnormality of mouse fetuses as the effects of methoxyacetic acid treatment.

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Abstract

This study is intended to characterize a protein that is linked with mouse limb teratogenicity as the effects of methoxyacetic acid (MAA) treatment. A single dose of MAA (10 mmol/kg body weight) was given by gavage on gestation day (GD) 11, whereas the control group were administered vehicle only. The pregnant mice were killed at 4 h after MAA treatment, and forelimb buds were isolated from both the control and treated group embryos. Proteins from forelimb buds GD 11 + 4 h, which were precipitated out using 40-60% ammonium sulfate, then were analyzed by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2-D SDS-PAGE) technique. The 2-D gels reveal one protein with 41.6 kDa and pI 6.4, which expression was downregulated after MAA treatment. Tentative protein identification via peptide mass database search and definitive protein identification via a primary sequence database search indicate that the protein matches exactly to 34/67 kDa laminin binding protein (LBP; P14206, SwissProt), which is encoded by p40 gene (MGI:105381). The identity was further verified by Western blotting with an antibody against the 67 kDa LBP. The results suggest that MAA treatment to pregnant mice downregulates the LBP-p40 in the forelimb buds.

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The Laminin Binding Protein p40 Is Involved in Inducing Limb Abnormality of Mouse Fetuses as the Effects of Methoxyacetic Acid Treatment

Aceng Ruyani¹*, Sri Sudarwati², Lien A. Sutasurya³, Sony H. Sumarsono⁴ and Torsten Gloe⁵
* Author AffiliationsReceived January 21, 2003.
Accepted May 23, 2003.

Abstract

This study is intended to characterize a protein that is linked with mouse limb teratogenicity as the effects of methoxyacetic acid (MAA) treatment. A single dose of MAA (10 mmol/kg body weight) was given by gavage on gestation day (GD) 11, whereas the control group were administered vehicle only. The pregnant mice were killed at 4 h after MAA treatment, and forelimb buds were isolated from both the control and treated group embryos. Proteins from forelimb buds GD 11 + 4 h, which were precipitated out using 40–60% ammonium sulfate, then were analyzed by two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2-D SDS-PAGE) technique. The 2-D gels reveal one protein with 41.6 kDa and pI 6.4, which expression was downregulated after MAA treatment. Tentative protein identification via peptide mass database search and definitive protein identification via a primary sequence database search indicate that the protein matches exactly to 34/67 kDa laminin binding protein (LBP; P14206, SwissProt), which is encoded by p40 gene (MGI:105381). The identity was further verified by Western blotting with an antibody against the 67 kDa LBP. The results suggest that MAA treatment to pregnant mice downregulates the LBP-p40 in the forelimb buds.

Key words limb teratogenicity methoxyacetic acid 2-D SDS-PAGE LBP-p40

Methoxyethanol (ME; synonymous with ethylene glycol monomethyl ether) is extensively used in paints, textile dyes, printing inks, brake fluid (Syed and Hecht, 1998), and the manufacture of semiconductors (Hays *et al.*, 2000). ME was shown to induce paw anomalies, syndactyly and oligodactyly, with forepaws exhibiting greater susceptibility than hindpaws in CD-1 mice when a single dose of 250 or 500 mg ME/kg body weight (bw) is administered orally on gestation day (GD) 11 (Horton *et al.*, 1985). Greene *et al.* (1987) also investigated the effects of ME in the forelimb bud of the CD-1 mouse embryo. Their results indicate that cell death is induced in the mesenchymal tissue and, to some extent, in apical ectodermal ridge (AER) after administration of 350 mg ME/kg bw to the dam on GD 11. The teratogenic effects of ME have been attributed to its primary metabolite, methoxyacetic acid (MAA), which is catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase in the rat dam's liver (Cheever *et al.*, 2001). MAA and ME have similar biological activities in mice; therefore their dose–response characteristics for producing developmental toxicity are nearly indistinguishable (Sleet *et al.*, 1988).

Several investigators reported that, after a single dose of MAA (10 mmol/kg bw), more than 94% of fetal limbs showed abnormalities, such as ectrodactyly, brachydactyly and syndactyly, when the substance was administered on GD 11 in Jcl:ICR (Rasjad *et al.*, 1991b), A/J (Sudarwati *et al.*, 1993), and Swiss Webster (SW; Suripto *et al.*, 1996) mice. The limb abnormalities are caused mainly by intensive cell death in the mesoderm of the limb plate (Rasjad *et al.*, 1991a; Sudarwati *et al.*, 1995; Suripto *et al.*, 1996). The AER also plays a role in inducing limb abnormalities, because it regresses rapidly in MAA-treated limbs compared to the control (Sudarwati *et al.*, 1995). It is well established that AER function is crucial for stimulating the proliferating zone of mesenchymal cells. A single dose of MAA (10 mmol/kg bw) given by gavage on GD 11 of SW mice caused a decrease of limb weight, total protein, and DNA content at GD 11 + 6 h, + 12 h, + 24 h, and + 48 h (Martgrita, 1998). Following a similar experiment design, we could show earlier that, in crude proteins from forelimb buds of GD 11 + 2 h, + 4 h, + 6 h, + 8 h, + 10 h, and + 12 h, one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (1-D SDS-PAGE) protein bands within the range of 29–45 kDa were already decreased after 4 h of MAA administration compared to the controls (Ruyani *et al.*, 2001). We therefore conducted this study to further investigate the identity of such regulated proteins.

MATERIALS AND METHODS

Experimental animals.

SW mice were used as experimental animals. Rearing the animals was done in a room at 23–27°C and 83% humidity. Food and water were given *ad libitum*. When female mice achieved their sexual maturity (10–12 weeks old) they were mated with a male (1:1). A vaginal plug detected the following morning was defined as day 0 of gestation (Sudarwati *et al.*, 1995).

Materials, dosage, and sample collection.

MAA (CH₃CH₂COOH) in liquid form (Product Number: 135-07762) was produced by Wako Pure Chemical Industries, Ltd. Japan. MAA diluted with sterilized distilled water was administered by gavage at a dose of 10 mmol/kg bw on GD 11. The control animals received sterilized distilled water (Sudarwati *et al.*, 1995).

The pregnant mice were killed by cervical dislocation at 4 h after MAA treatment (Ruyani *et al.*, 2001). Forelimb buds were isolated and were stored at –85°C until final analysis.

Protein precipitation.

Forelimb bud samples were homogenized at 4°C in 3 volumes 40 mM sodium phosphate, pH 8. Cellular debris was separated by centrifugation at 4,350 × g, 4°C, for 20 min (Beckman J2–HS). The finally obtained supernatants were isolated as crude-extract proteins.

Proteins of one part of the crude-extracts were precipitated for 30 min using 40–60% ammonium sulfate at 4°C. Precipitates were collected by centrifugation at 17,400 × g, 4°C, for 20 min (Beckman J2–HS; Bollag and Edelstein, 1991). The protein fraction was solubilized completely with 40 mM sodium phosphate, pH 8, dialyzed on cellulose membranes (Klotz, 1989), and stored at –20°C as samples for two-dimensional electrophoresis.

Two-dimensional electrophoresis.

The two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2-D SDS-PAGE) technique was applied according to Dunbar *et al.* (1990). The separation of proteins in the first dimension was performed according to their pI using two kinds of ampholytes (pH 3.5–10 and pH 5–7) followed by separation in the second dimension according to their molecular weight using the SDS-PAGE (Gestern, 1996). The 2-D electrophoresis was performed in mini format. Control and treated protein samples, each of 75 µg (Bohring and Krause, 1999; Sarto *et al.*, 1999), were electrophoresed in accordance to the instruction manual (BioRad).

Before running the second dimension, the tube gels were equilibrated in SDS buffer for 15 min. The tube gels were transferred onto a separating gel in mini format with 12% polyacrylamide, and a low molecular weight protein marker (6.5–66.0 kDa) was used as the standard. The gels were run at 120 V and finally stained with Coomassie Brilliant Blue R-250 (CBB R-250; Merrill, 1990).

Mass spectra and internal sequence analysis.

Excised spots from 2-D SDS-PAGE gels were digested with trypsin and extracted according to the procedures described by Fernandez *et al.* (1998). Extracted peptides were dried and solubilized in 10 ml of 0.1% TFA. One μ l of the 10 μ l preparation was for mass spectra analysis, and the remaining 9 μ l went to protein purification.

Mass spectra analysis was performed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF-MS) technique. One ml of the preparation was deposited on the sample probe of a Voyager Elite mass spectrometer (PerSeptive Biosystems) operated in the delayed extraction and reflection modes. The spectra were internally calibrated using the matrix dimer (379.09), and one of the trypsin autolytic peaks (2163.05; 2273.15; Fernandez *et al.*, 1998). Peptide masses were used for tentative protein identification via a peptide mass database search (ProFound; <http://www.rockefeller.edu>).

The remaining 9 μ l of the preparation was purified by high-performance liquid chromatography (HPLC) technique. One of the HPLC purified peptides was determined as a sample for determining amino acid (AA) internal sequence. The internal sequence was obtained through Edman sequence analysis (Fernandez *et al.*, 1998), and it could be used for tentative protein identification via a primary sequence database search (BLAST; <http://pdtc.rockefeller.edu>).

Western blotting.

Equal amounts of control and treated crude-extract-proteins were separated in one-dimensional (1-D) SDS-PAGE and were then transferred onto PVDF membrane. Non-specific antibody binding to the PVDF membrane was blocked with 2% gelatin in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20) at room temperature for 1 h (Kim *et al.*, 2000). The blot was then incubated using an antibody against the 67 kDa LBP (Cloe *et al.*, 1999) at dilution 1:1000 as the primary antibody. Antigen-antibody reaction was detected by anti IgG rabbit-alkaline phosphatase conjugate (Sigma) at dilution 1:7500 as the secondary antibody (Davis *et al.*, 1994).

RESULTS

Both control and treated forelimb buds were collected from each of 65 pregnant mice. The gained 2-D gels indicate that the appearance level of a protein with 41.6 kDa and pI 6.4 decreases significantly in the forelimb buds after MAA treatment (Fig. 1), which was obtained from 10 replicates. This significant difference of protein pattern between the control and treated groups is used as consideration for determining that the protein content might be influenced by MAA treatment. Further protein characterization was performed using MALDI TOF-MS and AA internal sequence analysis.

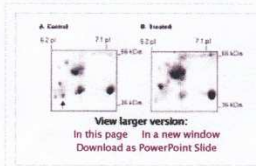


FIG. 1. Two-dimensional SDS-PAGE of the protein fraction of 40–60% ammonium sulfate extraction from Swiss Webster mouse forelimb buds GD 11 + 4 h. Detection was done by CBB R-250 staining. The arrow indicates a protein with 41.6 kDa and pI 6.4 decreased in expression in the treated group after MAA administration.

MALDI TOF-MS spectra were obtained from tryptic digestion of the protein with 41.6 kDa and pI 6.4 (Fig. 2). Twenty-seven peptide masses are applied for tentative protein identification via a peptide mass database search by the ProFound program. The search result details reveal that AA sequence of the matched peptide masses cover 29% of the protein sequence, and that the protein is ribosomal protein RS.40 K (Table 1).

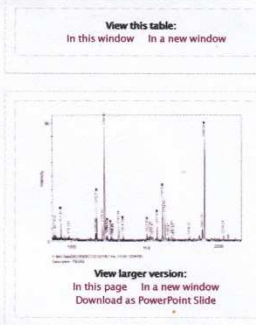


TABLE 1
Amino Acid Sequence of Matched Peptides from Tryptic Digestion of the Protein with 41.6 kDa and pI 6.4, and a Candidate for the Protein According to the ProFound Search Results

FIG. 2. MALDI TOF-MS spectra obtained from tryptic digestion of the protein with 41.6 kDa and pI 6.4. Stars show matched ones identified by ProFound program, and the rest are unmatched peptides.

For definitive protein identification, the digested protein mixture was purified using HPLC technique. One of the HPLC purified peptides was sequenced using Edman microsequencer at 12 cycles. The result reveals XXDXIYINLKR as internal sequence. For definitive protein identification, this internal sequence was compared with the primary sequence database (NCBI) search using the BLAST program. The definitive identification indicates that the protein with 41.6 kDa and pI 6.4 matches exactly to ribosomal protein RS.40K (laminin receptor; A29395, NCBI; Table 2; Fig. 3), which contains 295 AA.

TABLE 2
Definitive Identification of the Protein with 41.6 kDa and pI 6.4 via a Primary Sequence Database Search Using the BLAST Program

1	MSKLSLQK	MSKLSLQK	MSKLSLQK	MSKLSLQK	MSKLSLQK
2	MSKLSLQK	MSKLSLQK	MSKLSLQK	MSKLSLQK	MSKLSLQK
3	MSKLSLQK	MSKLSLQK	MSKLSLQK	MSKLSLQK	MSKLSLQK
4	MSKLSLQK	MSKLSLQK	MSKLSLQK	MSKLSLQK	MSKLSLQK

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FIG. 3.
Sequence position of the peptide fragment with 1418.76 Dalton on the original sequence (295 AA) of ribosomal RS.40 K-mouse with accession number A29395 (NCBI).

Homology searches reveal that RS.40K is 99.3% homologous to laminin binding protein (LBP) 34/67 kDa (P14206, SwissProt). It is known that the LBP is encoded by p40 gene at 71.0 centiMorgans of chromosome 9 in the mouse (MGI:105381; <http://www.informatics.jax.org>). Furthermore, Western blotting with the antibody against the 67 kDa LBP indicates that MAA treatment to pregnant mice causes a decrease of the LBP expression (42 kDa) in the forelimb buds (Fig. 4), which was obtained from five replicates.

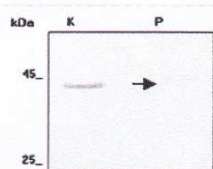


FIG. 4.
Western blotting with the 67 kDa LBP-specific antibody. Arrow indicates that MAA treatment to pregnant mouse cause to decrease LBP expression (42 kDa) in the forelimb buds. K: control; P: treated

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DISCUSSION

The present study, using a proteomics approach, reveals that the protein with 41.6 kDa and pI 6.4 is significantly decreased at 4 h after MAA treatment. Definitive protein identification indicates that the protein matches exactly to ribosomal protein RS.40K (laminin receptor; A29395, NCBI; Table 2; Fig. 3) and is 99.3% homologous to LBP-p40 (P14206, SwissProt; MGI:105381; Fig. 4). LBP-p40 is distributed on the nucleus, 40S ribosome, and the cell surface (Sato *et al.*, 1999). It is reported that LBP-p40 appears to exhibit multiple functions in various aspects of cell growth, embryonic development, and cancer progression (Klein, 2001).

Previous investigations indicate that MAA treatment may interfere with the availability of purine and pyrimidine bases, which are expected to affect DNA and/or RNA synthesis, which in turn influence normal cellular proliferation and differentiation in the developing mouse embryos (Mebus and Welsch, 1989). Furthermore, the inhibition of RNA or protein synthesis in certain cells may increase apoptotic cell death (Ellis *et al.*, 1991; Umansky 1996). It is well established that DNA fragmentation is the preliminary event before apoptotic cell death (Evan and Littlewood, 1998; Quarrie *et al.*, 1995). Limb teratogenicity studies show that the MAA-induced limb abnormalities are caused mainly by intensive cell death in the mesoderm of the limb plates (Rasjad *et al.*, 1991a; Sudarwati *et al.*, 1995; Surtipto *et al.*, 1996). Meanwhile, Sudarwati *et al.* (1995) report that AER also plays a role in the development of the MAA-induced limb abnormality, because it regresses rapidly on treated limbs compared to the control. Recent histological examinations clarify that the MAA-induced cell deaths are apoptotic and apoptosis is predominant than necrosis (Surjono and Haryono, 2002).

Kaneda *et al.* (1993), as well as Sato *et al.* (1996), have clarified that LBP-p40 is associated with both the nuclear envelope and chromatin DNA in interphase nuclei, while it is bound only to chromatin DNA in mitosis. Kinoshita *et al.* (1998) state that association of LBP-p40 with histones H2A, H2B, and H4 confers tight binding of LBP-p40 to chromatin DNA in the nucleus. Chromatin DNA may become unstable by the loss of LBP-p40 as a chromatin anchoring protein. Kaneda *et al.* (1998) suggest that the loss of LBP-p40 can induce apoptosis in HeLa cells. Therefore, MAA treatment that causes the decline of LBP-p40 production can lead to DNA fragmentation and then induce intensive apoptotic cell death in the mesoderm of the limb plate.

Auth and Brawerman (1992) state that LBP-p40 from mouse cells is associated with ribosomes and polysomes. According to Sato *et al.* (1999), the localization of LBP-p40 on the 40S ribosome is well conserved in a broad range of eukaryotes. As a part of the 40S ribosome, LBP-p40 is one of the key components of the protein synthetic machinery (Klein, 2001). The decrease of LBP-p40 in this experiment is predicted to inhibit protein synthesis and induce the intensive apoptotic cell death in the mouse forelimb buds. Furthermore, LBP-p40 is considered to be a precursor or a basic building block of the 67 kDa LBP (Buto *et al.*, 1998; Narumi *et al.*, 1999; Sato *et al.*, 1996; Satoh *et al.*, 1999; Tanaka *et al.*, 2000; Verlaet *et al.*, 2001). Landowski *et al.* (1995) indicate that the 67 kDa LBP might be a dimer of LBP-40, and that acylation may occur before or after dimerization. Meanwhile, Wang *et al.* (1992) report that overproduction of LBP-p40 in BHK cell increases the production of the 67 kDa LBP at the cell surface.

In the early stage of chick limb development, a 67 kDa laminin binding protein gene (cLbp) is expressed in the mesodermal region of the limb plate and in the AER. The 67 kDa LBP has a role in maintaining the PZ mesoderm in an undifferentiated state during the development of the chick limb bud (Hara *et al.*, 1997). This protein might serve as an auxiliary molecule involved in regulating the interaction of laminin with the $\alpha 6 \beta 6$ or $\alpha 6 \beta 1$ integrins (Klein, 2001). It is suggested that $\alpha 3$ and $\alpha 6$ integrins are required for the organization or compaction of presumptive AER cells into a distinct differentiated structure (Arcangelis *et al.*, 1999). The facts indicate that the 67 kDa LBP is required for the normal growth of AER. Therefore, MAA treatment that decreases the LBP-p40 expression may decrease the 67 kDa LBP production in the AER. The decline of LBP-p40 expression indirectly causes the degradation the AER in the limb buds. Sudarwati *et al.* (1995) report that the AER regresses rapidly on the MAA-treated limbs compared to the control. The alteration of AER properties can lead to reduction in the proliferation of the underlying mesenchymal cells.

It can be concluded that the decline of LBP-p40 expression after MAA treatment stimulates both the intensive apoptotic cell deaths in the mesoderm of the limb plate and the AER regression, which induces limb abnormality of the mouse fetuses.

Acknowledgments

A Graduate Team Research Grant, University Research for Graduate Education (URGE) Project Batch III, supported this study. Protein sequence analysis was provided by the Rockefeller University Protein/DNA Technology Center, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment, and with the help of Joseph Fernandez.

Footnotes

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Online ISSN 1096-0929 - Print ISSN 1096-6080

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