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Analysis of the Expression Pattern of Ebp1, an ErbB-3-Binding Protein

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Ebp1, a member of the *PA2G4* family, was isolated as an ErbB-3-binding protein in our laboratory using yeast two hybrid analysis. Although Ebp1 mRNA is ubiquitously expressed, little is known about either the expression of Ebp1 protein *in vivo* or its translation initiation site. Western blotting analysis of a wide range of cell lines and primary tissue indicated that in the majority of cases Ebp1 is expressed as a single protein which migrates at 48 kDa in SDS–polyacrylamide gels. We show using epitope-tagged expression constructs that the second, not the first, in-frame ATG is used for the initiation of translation of the endogenous protein, encoding a protein predicted to be 41.5 kDa. The molecular mass of endogenous Ebp1 protein derived from mouse liver and brain was determined by mass spectrometry and the data confirm that translation of endogenous Ebp1 in tissues is initiated from the second in-frame ATG. © 2001 Academic Press

Key Words: **Ebp1; ErbB-3; signal transduction; translation initiation; SELDI mass spectrometry.**

Ebp1, a member of the *PA2G4* family (1) was isolated as an ErbB-3-binding protein using yeast two hybrid analysis. Ebp1 interacts with the juxtamembrane domain of the ErbB-3 receptor in human breast cancer cell lines (2). Treatment of AU565 breast carcinoma cells with the ErbB-3 ligand heregulin results in translocation of Ebp1 from the cytoplasm to the nucleus (2). Ectopic expression of *ebp1* in ErbB-2, ErbB-3 expressing breast carcinoma cell lines results in inhibition of colony formation, an accumulation of cells in the G2/M phase of the cell cycle, and suppression of growth in soft agar (3).

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Ebp1 is the human homologue of a previously identified cell cycle regulated mouse protein p38-2G4 which migrates with molecular weight of about 38 kDa in SDS–PAGE (1). A cDNA encoding a predicted 44 kDa protein was isolated by Nakagawa *et al.* (4) from mouse ascites sarcoma cells that is identical to the p38-2G4 protein except for an additional 54 amino acids at its N terminal end. Although the predicted molecular weight of this protein is approximately 44 kDa, studies of Nagakwa *et al.* (4) indicated that this protein migrates at a molecular weight of 47 kDa. This protein is also identical to the one predicted by the cDNA isolated from human cells by La Martine *et al.* (5) and the Ebp1 cDNA isolated by our laboratory.

The presence of Ebp1 with different molecular weights isolated by different groups suggests the possibility that different isoforms of Ebp1 may exist *in vivo.* Data from our lab and others indicate that Ebp1 mRNA is ubiquitously expressed in both fetal and adult tissues of a wide variety of organisms. In addition, Ebp1 appears to be very conserved throughout evolution with homologues being present in yeast (6). Our laboratory has generated a series of antibodies against recombinant Ep1 produced from the full-length cDNA and Ebp1 peptides. As little work has been done to examine Ebp1 protein expression, we were interested in determining if isoforms of Ebp1 derived from different translational initiation sites might be present in different tissues.

The purpose of the current study was to characterize the expression of Ebp1 protein in different types of cell lines and primary tissues. We determined that in the majority of cell lines and tissues tested Ebp1 was present as a single protein migrating at 48 kDa in SDS gels. We show here that the second in-frame ATG was used for the initiation of translation. The molecular mass of endogenous Ebp1 protein was confirmed by mass spectrometry to be 42.2 kDa, conforming to the mass predicted using the second ATG.

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MATERIALS AND METHODS

Cell culture. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The cell lines were routinely cultured in RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO). Primary cultures of prostate and mammary epithelial cells were obtained from Clontech (San Diego, CA) and maintained as per the manufacturer's instructions.

Plasmids. A cDNA containing 150 bp of the 5′UTR and the complete coding region of Ebp1 (GenBank Accession No. U87954) was cloned into pcDNA3 using *Bam*H1–*Eco*R1 restriction sites. cDNA encoding a flag-tagged Ebp1 was also cloned into CMV2 (Sigma, St. Louis, MO) using *Eco*RI–*Bam*HI restriction sites to produce Ebp1 initiated from the first or second potential translation initiation sites. For this, the nucleotide sequences 196–1380 or 262–1380 were amplified using PCR with primers that incorporated the restriction sites. The fragments generated by PCR amplification were digested by the appropriate restriction enzymes, and the inserts purified from agarose gels after electrophoresis and ligated into the CMV2 vector. The orientation and sequence integrity of the cDNA inserts were confirmed by automated DNA sequencing in the core laboratory of the University of Maryland School of Medicine.

Immunoprecipitation and immunoblotting. For Western blot analysis, cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid sodium salt, 0.1% SDS, 100 μg/ml PMSF, 1 mM DTT, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin). Protein concentration was determined using a Bio-Rad detergent compatible kit (Bio-Rad, Richmond, CA). For each sample, approximately 30 μg of total protein extract was directly loaded onto SDS–polyacrylamide gels in Laemmli sample buffer. Proteins were electrotransferred onto PVDF membranes (Millipore Corp, Bedford, MA) and blotted with the Ebp1 antibodies described below, and horseradish peroxidase conjugated secondary antibodies. Blots were visualized using an ECL detection system (Amersham, Arlington Heights, IL). The rabbit polyclonal antibody prepared against an N terminal peptide corresponding to amino acids 54–67 predicted using the full-length cDNA (7) has been previously described.

A new polyclonal antibody generated against His-tagged recombinant Ebp1 was generated as follows. The cDNA encoding a recombinant His-tagged Ebp1 fusion protein was cloned into pQE31 (Qiagen, Valencia, CA) and transformed into the *E. coli* host stain M15. The 6xHis tagged fusion protein was purified from the bacterial lysates using Ni^+/nitrilotriacetic acid agarose according to the instructions provided by the manufacturer (Qiagen). The antiserum was generated in New Zealand white rabbits at Lampire Biologicals (Pipersville, PA). To purify the anti-Ebp1 antibody, His-Ebp1 proteins were conjugated to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ). The antibody was purified by affinity chromatography using His-Ebp1 linked Sepharose gel as previously described (8).

Isolation of Ebp1 from mouse tissues. Liver, kidney, and brain (approximately 1 g) from adult BALB/c mice (18–20 g) were homogenized in PBS (pH 7.4) using a Polytron homogenizer. Lysates were centrifuged at 500*g* for 15 min. The supernatant was then further centrifuged at 100,000*g* for 60 min using a Beckman ultracentrifuge (SW52 rotor) into membrane and cytoplasmic fractions. The membrane fraction was solubilized in PBS-1% Triton X-100 buffer containing protease inhibitors. The supernatants were then incubated with 1 ml of Sepharose beads containing 300 μg of αEbp1 antibody at 4°C overnight. After 3 × 5 ml washes with 150 mM NaCl, pH 4.5 (HCl), proteins were eluted from beads by incubating with 150 mM NaCl, pH 2.5 (HCl). The affinity purified proteins isolated from these fractions were electrophoresed on 10% SDS–polyacrylamide gels. Gels were silver stained using a SilverQuest Staining kit (Invitrogen, Carlsbad, CA) or subjected to Western blot analysis.

Identification of proteins using surface-enhanced laser desorption/ionization time of flight (SELDI) mass spectrometry. Aliquots (2 × 2 μl) of fractions of affinity-purified Ebp1 were spotted onto an eight spot H4 hydrophobic chip (Ciphergen Biosystem, Palo Alto, CA). The chips were incubated briefly at room temperature and then spotted with 1 μl of a saturated solution of alpha-cyano-4-hydroxycinnamic acid (50% acetonitrile, 0.2% trifluoracetic acid) and allowed to dry. The chips were analyzed by the SELDI-TOF technique (Ciphergen SELDI Protein Biology System I). Mass identification was performed averaging at least 200 laser shots of various regions of the Protein Chip surface. Only molecular weight peaks whose intensities had signal to noise ratio of >5 were designated as protein peaks (10). External standards were used for calibration.

RESULTS

Expression of Ebp1 in Cell Lines and Primary Cells

As different molecular weights for the Ebp1 (PA2G4) protein have been reported, we first examined the expression of Ebp1 protein using the anti-Ebp1 antibody. We had previously reported that endogenous Ebp1 protein derived from AU565 human breast carcinoma cells and COS7 monkey fibroblasts migrated as a single band at a molecular weight of approximately 50 kDa. To determine whether different isoforms of Ebp1 derived from different translation initiation sites might be expressed in other tissue types, we examined Ebp1 expression in a wide range of cell lines. In the majority of cell lines examined, Ebp1 migrated as a single band with a M_r of approximately 48 kDa. Ebp1 was expressed primarily as a 48 kDa protein in MCF-7, AU565, MDA-MB-453 (Fig. 1A), and MDA-MB 231 (data not shown) breast cancer cell lines. Other ErbB expressing tumor cells lines such as the ovarian cell line SKOV-3 (data not shown) and prostate carcinoma cell lines PC-3, DU-145 (Fig. 1A) and LNCaP (data not shown) also expressed Ebp1 which migrated as a single band at approximately 48 kDa. Of interest, Ebp1 protein was detected as a single 48 kDa band in the hematopoietic cell lines DAMI (megakaryoblast leukemia), HL-60 (promyelocytic leukemia), KG-1 (hairy cell leukemia), and K562 (erythoblastic leukemia) (Fig. 1A) which do not express the ErbB receptors (11). Ebp1 was also highly expressed in normal human prostate and mammary epithelial cell primary cultures (Fig 1B). We failed to detect Ebp1 protein only in MCF-10A non malignant human breast epithelial cells (1C).

We were then interested in examining protein expression of Ebp1 in primary tissues using an antibody to an N terminal peptide of Ebp1 (7). We first found that the Ebp1 antibody was able to recognize a 48 kDa protein in both murine fibroblasts (NIH 3T3) and immortalized murine mammary epithelial cells (C 127) (Fig. 2A). We next found that Ebp1 was expressed as a single 48 kDa protein in mouse liver, brain, kidney and lung (Fig. 2B).

Translation of Ebp1 is initiated at the second ATG. As mentioned, the longest ORF in the Ebp1 cDNA encodes a protein with a predicted molecular weight of

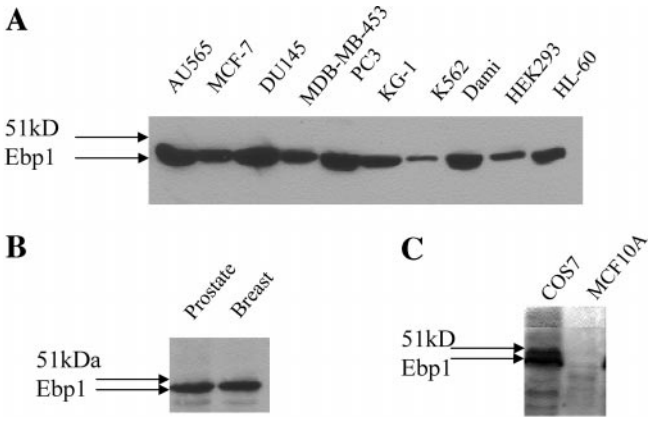

FIG. 1. Western blot analysis of Ebp1 protein expression. Cell lysates derived from cultured cell lines were prepared as described under Materials and Methods. Equal amounts (30 μg) of protein were subjected to SDS–PAGE (10%). Western blot analysis was then performed with the use of an antibody directed against an N terminal Ebp1 peptide. (A) Lysates of cultured cell lines: breast carcinoma cell lines: AU565, MCF-7, MDA-MB 453; prostate carcinoma cell lines: DU145, PC3; hematopoietic cell lines KG-1, K562, Dami, HL-60; human embryonic kidney HEK 293. (B) Primary cultures of human mammary or prostate normal epithelial cells. (C) MCF10A cells do not express Ebp1. Lysates of COS7 and MCF10A cells were resolved by SDS–PAGE and immunoblotted with an antibody to full-length Ebp1 (2). The filter was deliberately overexposed to detect Ebp1 protein.

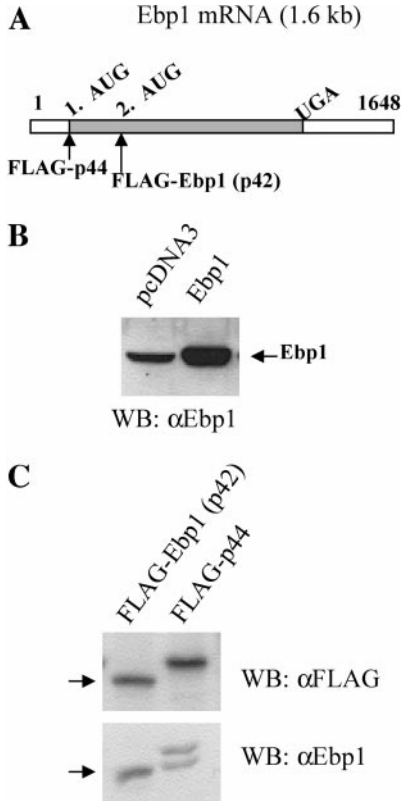

FIG. 3. Ebp1 protein is initiated from the second in-frame ATG. (A) Diagram of the complete Ebp1 mRNA. (B) A pcDNA3 vector encoding the complete Ebp1 cDNA (Ebp1) or the empty vector (pcDNA3) was transfected into COS7 cells. Cells were harvested 48 h later and proteins analyzed by Western blotting using an antibody to Ebp1. (C) Plasmid DNAs encoding FLAG tagged p44Ebp1 (FLAG-p44) or p42Ebp1 (FLAG-Ebp1) were transfected into COS7 cells as described under Materials and Methods. Forty-eight hours later, cell lysates were prepared and proteins (20 μg) analyzed by Western blotting using either αFLAG or αEbp1 antibody as indicated.

43,812.67 kDa. A second in-frame ATG, 66 bp downstream from the first ATG, could also be used as a start codon encoding a protein of a predicted molecular weight of 41270.90 kDa (Fig. 3A). Thus, either ATG could encode the 48 kDa protein observed in cultured cells. To determine the M_r of the protein produced by the full-length cDNA, pcDNA3 containing 150 bp of the 5′UTR and the complete coding region of Ebp1 (see Materials and Methods) was transfected into COS7 cells. Only one band with a molecular weight of about 48 kDa was observed in whole cell lysates using the αEbp1 antibody by Western blot analysis (Fig. 3B). This band was of increased intensity compared to the band detected in cells transfected with pcDNA3 alone. This finding suggests that the 48 kDa protein was the major form of Ebp1 expressed from the transfected plasmid and of the same M_r as endogenous Ebp1 in COS7 cells.

We next determined the initiation codon used for the translation initiation of Ebp1 *in vivo*. We focused on the first two ATG initiation codons. For that purpose, we made two constructs encoding the protein initiated from either the first or the second ATG, both tagged with a FLAG sequence at the N-terminal end of the fusion proteins (Fig. 3C). Translation is initiated at an ATG upstream of the FLAG epitope. Both plasmids were transfected into COS7 cells and lysates were analyzed for the presence of the fusion proteins using an anti-FLAG antibody or the anti-Ebp1 antibody. Re-

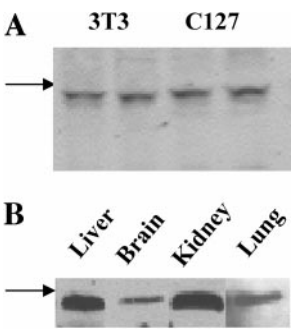

FIG. 2. Western blot analysis of Ebp1 protein expression in primary tissues. (A) NIH3T3 cells (mouse fibroblasts) or C127 (mouse breast carcinoma cells) were resolved by SDS–PAGE and immunoblotted for Ebp1 protein as described. (B) The indicated primary tissues were isolated from BALB/c mice and cell lysates prepared as described under Materials and Methods. Cytoplasmic membrane fractions were resolved by SDS–PAGE and analyzed by immunoblotting for Ebp1 protein as described. The arrows indicate the position of the 51 kDa marker.

sults showed that endogenous Ebp1 migrated at the same molecular weight (48 kDa) as the flag-tagged Ebp1 protein initiated from second ATG [FLAG-Ebp1 (p42), Fig. 3C]. The molecular weight of Ebp1 initiated from the first ATG (FLAG-p44) was about 52 kDa, bigger than both endogenous Ebp1 and the FLAG-Ebp1 initiated from the second codon. These results established that the translation of Ebp1 protein was initiated from the second ATG.

Determination of the Molecular Weight of Endogenous Ebp1 by Mass Spectrometry.

To determine the molecular weight of endogenous Ebp1 derived from primary cells, we decided to purify Ebp1 from mouse tissues. Data from immunostaining and biochemical analysis indicated that Ebp1 was enriched in the membrane fraction (data not shown). Therefore, we used this fraction as a source of protein to determine the molecular weight of endogenous Ebp1 in tissues. Ebp1 proteins were purified by immunoaffinity chromatography as described under Materials and Methods. Specifically bound proteins were either resolved by SDS–PAGE and visualized by silver staining or subjected to mass spectrometry. Analysis of SDS–polyacrylamide gels revealed a major band migrating around 48 kDa in liver, kidney, and brain lysates (Fig. 4A). Immunoblot analysis indicated that only the 48 kDa protein reacted with Ebp1 antibody (data not shown), suggesting that the 48 kDa protein band was indeed purified Ebp1. A number of other proteins copurified with Ep1 with varying intensities.

We next used time of flight mass spectrometry to determine the molecular mass of Ebp1. Aliquots of liver proteins were spotted on H4 hydrophobic chips and analyzed by SELDI mass spectrometry. Peaks at 35,410 and 42,171 dalton were identified (Fig. 4B), in agreement with data derived from the gels (Fig. 4A). Therefore, the molecular mass of endogenous Ebp1 was determined to be 42,171. These data confirm the transfection experiments suggesting that endogenous Ebp1 is initiated from the second ATG.

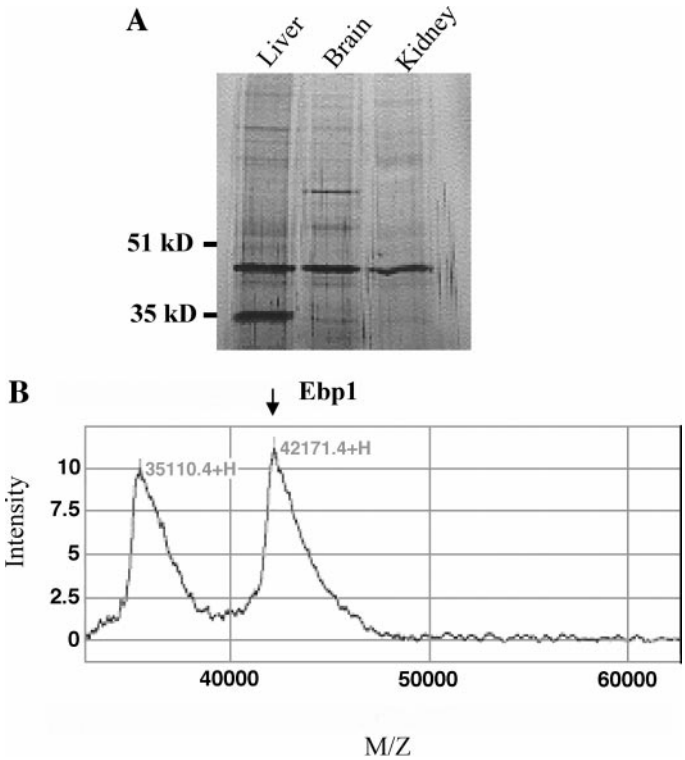

FIG. 4. Purification of Ebp1 complexes. (A) Endogenous Ebp1 was purified from mouse liver, kidney, and brain as indicated on Ebp1 immunoaffinity columns using an antibody prepared against His- tagged Ebp1. Eluates were separated by 10% SDS–PAGE, and proteins visualized by silver staining. (B) Detection of Ebp1 and Ebp1 binding proteins by SELDI mass spectrometry. Aliquots of fractions eluting at pH 2.5 from Ebp1 immunoaffinity columns were spotted onto the surface of H4 ProteinChip arrays and analyzed by SELDI- TOF mass spectrometry. The plotted spectra depict the relative peak intensities virus mass to charge ratios of proteins. The arrow indicates Ebp1.

DISCUSSION

We recently cloned Ebp1 via its interaction with ErbB-3. Ebp1 cDNA is expressed in a wide variety of adult and fetal tissues. Analysis of mRNA and protein sequences reveal that Ebp1 is conserved during evolution (6). The purpose of this paper was to begin to examine the range and pattern of expression of Ebp1 protein.

As the cDNA we (2) and others (5) have cloned contains three potential translation initiation sites with weak Kozak sequences (12), we first examined if different isoforms of Ebp1 were expressed in cultured and primary cells. We have found that one protein that migrates at 48 kDa on SDS polyacrylamide gels is the major form of Ebp1 expressed in most cell types. Ebp1 appeared to ubiquitously expressed. Of interest, Ebp1 was expressed in hematopoietic cell lines and primary cells, which do not contain any members of the ErbB receptor family (13). We also demonstrate that NIH3T3 murine fibroblast cells express Ebp1 protein, despite the apparent absence of expression of ErbB receptor family members1 (4). The mRNA and protein data lead one to speculate that Ebp1 may not function solely through the ErbB3 receptor. In fact, Ebp1 protein and mRNA was expressed in over 30 cell lines examined thus far, except for MCF10A immortalized nonmalignant breast epithelial cells. As normal breast epithelial cells expressed abundant amounts of Ebp1, the significance of the lack of Ebp1 expression in MCF10A cells is currently unclear.

We found using specific expression vectors that the major form of endogenous Ebp1 expressed appears to be initiated from the second, rather than the first, in-frame ATG of the Ebp1 cDNA. This finding was

confirmed by mass spectrometry indicating that endogenous Ebp1 derived from mouse liver is a protein of approximately 42,000 kDa. The biological significance of the use of the second translation initiation sites is unclear. It is possible that the production of a protein initiated from the first site might be deleterious for growth of the cells tested. We also cannot exclude the possibility that a protein is produced from the first ATG in some cells or under certain physiological conditions. Proteins translated from alternative translation initiation sites could perform different functions in different cell types (16).

The Ebp1 protein contains 12 potential phosphorylation sites and we have already shown that Ebp1 is phosphorylated *in vivo* (15). In addition, Ebp1 contains potential myristylation sites. The identification of a peak of endogenous Ebp1 with a molecular weight of 42 kDa by mass spectrometry analysis suggests that the majority of Ebp1 is modified as the predicted M_r is 41,270. We cannot exclude the possibility that Ebp1 is modified on alternative or additional sites under different conditions *in vivo.*

The 48 kDa form observed migrates anomalously from the protein predicted by the cDNA. Treatment of cell lysates with phosphatase does not increase the migration rate (7) indicating that the retarded migration is not due to phosphorylation. The structural properties of Ebp1 that account for its migration properties are currently unknown, as well as the relationship of such structure to Ebp1 function. Similarly, Nagakawa noted that the cDNA he isolated encoded a protein that migrated at around 47 KDa., although it had a predicted M_r of 44 kDa.

Biochemical analysis of Ebp1 in tissues indicated that the bulk of the protein sedimented at 100,000 g (data not shown) suggesting that it associated with cellular membrane structures. This is in keeping with our previous immunofluorescent data (7) that indicated a punctuate pattern of staining in the cytoplasm. We have not yet measured Ebp1 in the nucleus by mass spectrometry. It is possible that nuclear localized Ebp1 may have a different molecular weight due to differences in post translational modifications or the use of the first initiation codon. However, as proteins used in Western blotting included both nuclear and cytoplasmic fractions, it is unlikely that molecular weight of nuclear Ebp1 differs from that of Ebp1 found in the cytoplasm.

In summary, we have found that Ebp1 is ubiquitously expressed as a protein that migrates as a single band at 48 kDa in SDS gels. The second in frame translation initiation site of the cDNA is used for translation of the endogenous protein. Endogenous Ebp1 derived from mouse liver appears to be a 42,171 kDa protein as determined by mass spectrometry. The widespread expression of Ebp1 suggests that it may play a role in cellular processes in many types of tissue.

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