Identification and Molecular Cloning of a Human Selenocysteine Insertion Sequence-binding Protein

A BIFUNCTIONAL ROLE FOR DNA-BINDING PROTEIN B*

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Prokaryotic and eukaryotic cells incorporate the unusual amino acid selenocysteine at a UGA codon, which conventionally serves as a termination signal. Translation of eukaryotic selenoprotein mRNA requires a nucleotide selenocysteine insertion sequence in the 3'-untranslated region. We report the molecular cloning of the binding protein that recognizes the selenocysteine insertion sequence element in human cellular glutathione peroxidase gene (GPX1) transcripts and its identification as DNA-binding protein B, a member of the EFL/dbpB/YB-1 family. The predicted amino acid sequence contains four arginine-rich RNA-binding motifs, and one segment shows strong homology to the human immunodeficiency virus Tat domain. Recombinant DNA-binding protein B binds the selenocysteine insertion sequence elements from the GPX1 and type I iodothyronine 5’-deiodinase genes in RNA electrophoretic mobility shift assays and competes with endogenous GPX1 selenocysteine insertion sequence binding activity in COS-1 cytosol extracts. Addition of antibody to DNA-binding protein B to COS-1 electrophoretic mobility shift assays produces a slowly migrating “supershift” band. The molecular cloning and identification of DNA-binding protein B as the first eukaryotic selenocysteine insertion sequence-binding protein opens the way to the elucidation of the entire complex necessary for the alternative reading of the genetic code that permits translation of selenoproteins.

Both prokaryotic and eukaryotic cells incorporate the sulfur-like element selenium into polypeptides by the translational insertion of the unique amino acid selenocysteine. Most selenoproteins are enzymes that contain a single selenocysteine moi-
SECIS-binding Protein: Bifunctional Role for dbpB

The 1.4-kilobase pair dbpB cDNA into the His tag vector pRSET B (Invitrogen) was achieved by insertion of a SacI-PstI fragment of dbpB cDNA from pBluescript KS into pRSET B digested by SacI-PstI. Constructs of pBluescript KS (Stratagene) with inserts of wild type and mutant GPX1 SECIS sequences and pSP64 (Promega) carrying human immunodeficiency virus (HIV) trans-activation response (TAR) element were developed as described previously (14).

**Northern Blots**—Primary cultures of human monocyte-derived macrophages were prepared as described previously (21). Total cellular RNA was extracted from these and from HL-60 cells by differential precipitation from guanidine HCl (22) and processed by standard methods for Northern blot analysis (23).

**Overexpression of His-tagged dbpB Protein**—The plasmid pRSET B (Invitrogen) containing His-tagged dbpB cDNA was used to transform *E. coli* strain BL21(DE3) carrying the plasmid pLYsE, so that the expression of the fused gene was controlled by bacteriophage T7 RNA polymerase (24). The cells were grown at 37 °C to an *A*_{600} = 0.5–0.8, induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h, and then lysed by sonication. Recombinant His-tagged dbpB was purified by F chromatography according to the manufacturer's protocol (Qiagen, Inc.) and then further purified by electrophoresis on a 10% SDS-polyacrylamide gel.

**Production of dbpB-specific Antibodies**—Antibodies against a dbpB peptide were raised in rabbits immunized with a synthetic peptide corresponding to the C-terminal 15 amino acids of human dbpB (AENS-SAYAAGQGGA) coupled to keyhole limpet hemocyanin at an additional N-terminal tyrosine. Domain-specific anti-dbP antibodies were purified from rabbit serum by affinity chromatography on peptide-Affigel 10 affinity matrices and stored frozen at −70 °C until use. Rabbit antibody against the entire His-tagged dbpB protein was prepared (at Berkeley Antibodies, Inc., Berkeley, CA) by immunization of rabbits with recombinant protein. Concentrated rabbit polyclonal anti-dbB IgG was prepared by precipitation of rabbit serum with 40% ammonium sulfate, followed by resuspension in buffer K and concentration by Amicon (Micron) ultrafiltration. For affinity purification of anti-dbB IgG, an affinity column was prepared by coupling the His-tagged dbpB protein to cyanogen bromide-activated Sepharose 4B (Sigma) following standard procedures (25); then immunoaffinity purification of the antibody was accomplished by standard methods (25).

**Western Blot Assays**—Antibodies for primary probing included antibodies to dbpB as described above as well as a mouse monoclonal antibody (7T-Tag AP conjugate system; Novagen, Inc.) recognizing the 11-amino acid epitope coded by sequences in the multicloning site of the vector pRSET B (Invitrogen, Inc.). Western blots were performed according to the protocol of the ProtoBlot Western blot AP System (Promega). Briefly, protein samples were fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, membranes were incubated with antibody, in some cases in the absence or the presence of 5 μl of 10 mg/ml dbpB C-terminal 15-amino acid peptide or His-tagged dbpB protein. Detection of antibody-targeted protein bands was achieved by either alkaline phosphatase-mediated color development (Promega) or by horseradish peroxidase-mediated chemiluminescence (ECL, Amersham Corp.) after second antibody incubation with appropriately conjugated goat antibod- ies against rabbit IgG.

**Electrophoretic Mobility Shift Assays**—Gel shifts were performed as described previously (14). For “supershift” assays, 5–10 μl of concentrated, affinity-purified anti-dbB antibody was added to the gel shift assay mixture and incubated 20 min at 30 °C and then 20 min at 4 °C prior to loading on the gel.

**Results**

**Expression Library Screening**—Two λgt11 cDNA expression libraries from human K562 and HeLa cell lines were screened with a [32P] labeled synthetic RNA transcript from the SECIS region of the human cellular glutathione peroxidase gene GPX1 (4). This RNA probe was identical to that used for detection and characterization of SECIS binding activity in COS-1 cell extracts (14, 26).

Approximately 107 plaque pheages were screened from each of the two libraries. Approximately 60 positive plaques were detected by binding of the radiolabeled GPX1 SECIS probe and specificity was established by secondary screening. For the secondary screening, two additional radiolabeled RNA probes were used as negative controls: a mutant GPX1 SECIS probe with a deletion of six nucleotides at the 3’ end and a probe containing the HIV TAR element found at the 5’ end of all nascent HIV-1 transcripts. The former probe was predicted by the FOLDRNA program (Genetics Computer Group, Inc., Madison, WI) to form an overall stem loop secondary structure identical to the native SECIS except for the loss of the second strand of the basal stem. The HIV TAR element also forms a stem loop (27) but with structural features distinct from that of the GPX1 SECIS. We had previously demonstrated by gel shift analysis that the SECIS-binding protein from COS-1 cells has a much higher affinity for the wild type than for the deletion mutant GPX1 SECIS probe and that it does not recognize the HIV TAR element (14). Therefore candidate plaques were selected by the following criteria: strong signal with the wild type GPX1 SECIS probe, weaker signal with the deletion mutant probe, and no signal with the HIV TAR probe.

After secondary screening, sequencing of phage clones from the positive plaques revealed segments of dbpB cDNA in four independent clones from the K562 cDNA library and three from the HeLa cDNA library. The largest cDNA clone, containing 1.4 kilobases, was identical to the published (17) dbpB transcript approximately 1.9 kilobase in length (data not shown). Overexpression of His-tagged dbpB protein—To characterize the GPX1 SECIS binding activity of dbpB, we prepared peptide-tagged dbpB with an N-terminal six-histidine epitope, overexpressed the construct in *E. coli*, and purified the His-tagged protein on a nickel-nitritotriacetic acid resin column. The purity and molecular mass were confirmed by Coomassie Blue staining of SDS-polyacrylamide gels (data not shown). The purified His-tagged protein specifically bound the GPX1 SECIS element, as demonstrated both by direct binding and by...
competition with the native binding activity in COS-1 cytosol extracts. In RNA electrophoretic mobility shift assays, illustrated in Fig. 2, incubation of the His-tagged dbpB with radiolabeled GPX1 SECIS RNA produced a major shift of probe to a position of slower migration through the gel (seventh lane). Furthermore, addition of increasing amounts of the His-tagged protein to the assay mixtures containing probe and COS-1 extract (third through sixth lanes) caused a steady decrease in the intensity of two cytosolic gel shift bands and a concomitant increase in the intensity of a single, newly formed band with a slightly slower migration rate.

In separate electrophoretic mobility shift assays (not shown), the purified His-tagged dbpB produced a gel shift with the SECIS from the rat type I iodothyronine 5'-deiodinase but did not interact with a GPX1 SECIS 6-nucleotide basal stem deletion probe or an HIV TAR probe, indicating that its binding is not strictly limited to the SECIS from GPX1 and that it specifically recognizes the functional form of the SECIS over non-functional or unrelated stem loop RNA structures.

**Demonstration of dbpB as a Cellular GPX1 SECIS-binding Protein**—Electrophoretic mobility shift assays (data not shown) confirmed that HeLa cytosol extract caused the same band retardation pattern on the nondenaturing polyacrylamide gel as that obtained with the COS-1 extract. Western blots (Fig. 3, first and second lanes) with affinity-purified polyclonal antibody raised against the C-terminal peptide of dbpB showed a single protein band of approximately 48 kDa of molecular mass in cytosol extracts from both cell types. Preincubation of the antibody with the C-terminal peptide from dbpB completely eliminated the bands (Fig. 3, third and fourth lanes). The estimated size of the immunoreactive protein exceeds that predicted by the cDNA sequence and expressed in E. coli, a discrepancy most likely due to either post-translational modification in mammalian cells or truncation of the dbpB cDNA during the library construction.

To establish that dbpB contributed directly to the RNA gel shift bands produced by incubation of GPX1 SECIS probe with COS-1 cell extracts, we tested whether addition of antibody to dbpB would produce a supershift due to further retardation of electrophoretic mobility by attachment of antibody to the protein-RNA complex. As shown in Fig. 4, addition of concentrated rabbit polyclonal anti-dbpB IgG raised against recombinant His-tagged dbpB resulted in the appearance of a slowly migrating supershifted band (lane 4) and in some experiments (not shown) the diminution of intensity of both gel shift bands. Addition of preimmune IgG did not produce any supershift (lane 3) nor did addition of the immune IgG without COS-1 extract (lane 6). Similar results were obtained with affinity-purified rabbit polyclonal anti-dbpB antiserum (not shown).

These results indicate that dbpB-SECIS interactions contribute to the gel shift observed with COS-1 cell extracts, which we have previously demonstrated to represent specific binding to the GPX1 SECIS element (14).

**DISCUSSION**

The molecular cloning of a human SECIS-binding protein by screening a cDNA expression library with a radiolabeled GPX1 SECIS RNA probe reveals that dbpB specifically binds to the RNA structure essential for translation of selenoproteins. Historically, as the name implies, dbpB was first identified as a DNA-binding protein of unknown specificity and was cloned from a human placenta expression library using DNA fragments from an enhancer region of the human epidermal growth factor receptor gene and the promoter region of the human c-erb-2 gene (17). dbpB is a member of the EFL1/dbpB/YB-1 family of DNA-binding proteins, whose members share, along with DNA-binding protein A (30), a highly conserved region of 100 amino acids highly homologous to the E. coli cold shock domain (31). Although dbpB has previously had no known function, it binds to the leukosialin (CD43) promoter (30); in addition, a binding protein clone “BP-8,” which is virtually identical to dbpB, binds two functionally significant homopy-
rimidine sites that form intramolecular triplex (H-DNA) structures upstream from the human \( \gamma \)-globin genes (19). Enhancer factor IA is a trans-acting factor that binds to the Rous sarcoma virus long terminal repeat enhancer and promoter at two inverted CCAAT box motifs (32). The Y box-binding members of the family include YB-1, a protein that recognizes the Y box (inverted CCAAT motif) of the HLA-DR \( \alpha \) chain gene (18), as well as two Xenopus CCAAT-binding transcription factors, FRGY1 and FRGY2 (33); the former is 83% homologous to dbpB.

At least two members of the EFL\(_d\)/dbpB/YB-1 family also bind RNA and are involved in translational regulation. FRGY2 binds RNA at basic regions distinct from the cold shock domains (34). The major core protein of cytoplasmic messenger ribonucleoprotein particles (p50) is also a member of the Y box family (35) and appears to function primarily as a nonspecific, probably translationally inhibitory, RNA-binding protein.

Many RNA-binding proteins are bifunctional, with other roles including transcriptional regulation, like the EFL\(_d\)/dbpB/YB-1 family, as well as enzymatic activities. The homeodomain RNA-binding protein boid also recognizes and binds to discrete target sequences in the 3'-untranslated region of mRNA encoding the opposing homeodomain protein caudal, leading to its translational suppression (36, 37). The enzyme aconitase probably translationally inhibitory, RNA-binding protein.

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