Protein X, recently renamed dihydrolipoamide dehydrogenase-binding protein (E3BP), is required for anchoring dihydrolipoamide dehydrogenase (E3) to the dihydrolipoamide transacetylase (E2) core of the pyruvate dehydrogenase complexes of eukaryotes. DNA and deduced protein sequences for E3BP of the human pyruvate dehydrogenase complex are reported here. With the exception of only a single lipoyl domain, the protein has a segmented multi-domain structure analogous to that of the E2 component of the complex. The protein has 46% amino acid sequence identity in its amino-terminal region with the second lipoyl domain of E2, 38% identity in its central region with the putative peripheral subunit-binding domain of E2, and 50% identity in its carboxyl-terminal region with the catalytic inner core domain of E2. The similarity in the latter domain stands in stark contrast to E3BP of Saccharomyces cerevisiae, which is quite different from its homologous transacetylase in this region. The putative catalytic site histidine residue present in the inner core domains of all dihydrolipoamide acyltransferases is replaced by a serine residue in human E3BP; thus, catalysis of coenzyme A acetylation by this protein is unlikely. Coexpression of cDNAs for E2, E3, and E3BP resulted in the formation of an E2:E3:E3BP subcomplex that spontaneously reconstituted the pyruvate dehydrogenase complex in the presence of native E1 and recombinant pyruvate decarboxylase (E1).

The pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate with the formation of CO2, acetyl-CoA, and NADH. The eukaryotic complex has 30 copies of a tetrameric (αβ)2 pyruvate decarboxylase (E1) component noncovalently bound along the edges of an icosahedral 60 meric dihydrolipoamide acetyltransferase (E2) core (1). Twelve copies of a homodimeric dihydrolipoamide dehydrogenase (E3) component are believed held on the faces of the E2 core by a corresponding number of monomeric E3-binding proteins (protein X, E3BP) (2, 3). With the exception of E3BP, the role of each enzymatic component in the overall reaction catalyzed by the complex is basically understood. E1 catalyzes a thiamine diphosphate-dependent oxidative decarboxylation of pyruvate and the reductive acetylation of a lipoyl residue covalently attached to the lipoyl domain of E2. E2 then catalyzes transfer of the acetyl group to coenzyme A, leaving a reduced E2 lipoyl group that the E3 component uses as an electron source for FAD-dependent reduction of NAD+ to NADH.

Called protein X because its function was not apparent at the time it was originally discovered to be a component of eukaryotic PDCs (4–6), E3BP has generated considerable interest because of sequence similarity with E2 (7–10) and evidence that it has a covalently bound lipoyl moiety (4, 6, 8, 11). Its function was difficult to establish in early studies because of a very tight association with the E2 core of the complex. Very elegant limited proteolysis and immunological studies (12, 13) provided the first evidence that protein X contributes to the binding and function of E2. Cloning of the E3BP gene of Saccharomyces cerevisiae (14) revealed a protein structure that resembles the E2 component of yeast in its amino terminus but not the remainder of the molecule. Subsequent gene disruption studies provided definitive proof that protein X should be considered an E2-binding protein (15). Questions left unsettled included whether mammalian E3BP is completely analogous to yeast E3BP, whether the lipoyl moiety of mammalian E3BP is functionally important for catalytic activity of the complex, and whether the inner core of the mammalian E3BP has transacetylase activity.

The deduced amino acid sequence for the first mammalian (human) E3BP is presented here. Although similar in their amino-terminal, lipoyl-bearing domains, the mammalian E3BP and the yeast E3BP are markedly different in their carboxyl-terminal regions. Indeed, the human E3BP is more homologous to mammalian E2 throughout its primary sequence than it is to yeast E3BP. Previous evidence that the mammalian E3BP contains only a single lipoyl domain in its amino terminus (8), rather than the two tandemly arranged lipoyl domains characteristic of mammalian E2, is confirmed. The active site histidine residue characteristic of transacetylases of all α-ketoacid dehydrogenase complexes is not conserved in the mammalian E3BP.
EXPERIMENTAL PROCEDURES

cDNAs—Two clones found by searching of the expressed sequence tag data base of the National Center for Biotechnology Information (National Library of Medicine, Bethesda, MD) were analyzed in this study. HS8032 was identified by the presence of sequence encoding for the amino-terminal peptide of E3BP, and T77385 was identified by a sequence encoding an internal peptide of E3BP. The cDNA of HS8032 in pPDHE2 was 2,355 base pairs long; the cDNA of T77385 in Lamdfid vector is 1,965 base pairs long.

Sequencing—Sequencing of double-stranded plasmid DNA was done by the Biochemistry Biotechnology Facility (Indiana University) using Taq DyeDeoxy terminator cycle sequencing kit with AmphiTag DNA polymerase, (Perkin-Elmer) following manufacturer instructions. Both strands were sequenced.

Construction of a Bacterial Expression Vector for the Human E3BP Component of the Pyruvate Dehydrogenase Complex—To remove the mitochondrial targeting sequence and 3′-non-coding region from the cDNA for the human E3BP component (kindly provided as a generous gift by Dr. Mulchand Patel, SUNY at Buffalo, NY), NdeI and XhoI sites flanking the coding region were created by polymerase chain reaction (PCR) with Pfu DNA polymerase (Strategene). The sense antisense primer pair corresponded to bases 162–183 and 1827–1850 of human E3BP cDNA (CAT ATG AGT CTT CCC CCG CAT CAG AAG GTT CC(A/C)TC). The fidelity of constructs was established by nucleotide sequencing.

The expression cassette for human E3BP (carrying T7 promoter, ribosome-binding site, cDNA for E3BP component, and T7 terminator) was cut from pPDHE223a with BglII and NdeI. Resulting DNA was purified, blunt-ended with T4 DNA polymerase, and ligated into the pACYC vector (New England Biolabs) cut with HindIII and blunt-ended with T4 DNA ligase. Ten clones of pPDHE223a were transformed into competent TG-1 cells. Transformants were selected on YT agar plates containing 35 μg/ml of chloramphenicol. Colonies that expressed chloramphenicol resistance were screened for the presence of inserts by PCR with the above primers.

Construction of a Bacterial Expression Vector for Human E2/E3BP Subcomplex of the Pyruvate Dehydrogenase Complex—Ncol and EcoRI restriction sites flanking the coding region of E3BP cDNA were constructed by PCR with Pfu DNA polymerase. The sense, Ncol containing, primer (TGG CCC ATG GTAT CCC CTT ATT AAG ATC ATG ATG) corresponds to bases 168–191 of the E3BP cDNA. Antisense primer (CTT GAA TTC CTA GGC AAG TCC GAG GTG ATT CT) corresponds to bases 1492–1514 of the E3BP cDNA and contains an EcoRI restriction site. Resulting cDNA of approximately 1.3 kilobases was digested with NcoI/EcoRI and ligated between the corresponding sites of plasmid pET-23a (Novagen). The fidelity of constructs was established by nucleotide sequencing.

Partial Purification of the Recombinant Proteins—The fidelity of constructs was established by nucleotide sequencing.

The expression cassette for human E3BP (carrying T7 promoter, ribosome-binding site, cDNA for E3BP component, and T7 terminator) was cut from pPDHE223a with BglII and NdeI. Resulting DNA was purified, blunt-ended with T4 DNA polymerase, and ligated into the pACYC vector (New England Biolabs) cut with HindIII and blunt-ended with T4 DNA ligase. Ten clones of pPDHE223a were transformed into competent TG-1 cells. Transformants were selected on YT agar plates containing 35 μg/ml of chloramphenicol. Colonies that expressed chloramphenicol resistance were screened for the presence of inserts by PCR with the above primers.

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The expression cassette for human E3BP (carrying T7 promoter, ribosome-binding site, cDNA for E3BP component, and T7 terminator) was cut from pPDHE223a with BglII and NdeI. Resulting DNA was purified, blunt-ended with T4 DNA polymerase, and ligated into the pACYC vector (New England Biolabs) cut with HindIII and blunt-ended with T4 DNA ligase. Ten clones of pPDHE223a were transformed into competent TG-1 cells. Transformants were selected on YT agar plates containing 35 μg/ml of chloramphenicol. Colonies that expressed chloramphenicol resistance were screened for the presence of inserts by PCR with the above primers.

Two of these clones (HS8032 and T77385) were sequenced and found identical to one another except that T77385 is 370 base pairs shorter at its 5′ end. Clone HS8032 has an open reading frame of 1503 base pairs that encodes a protein of 501 amino acid residues with a calculated molecular weight of 54,085 (Fig. 1).

RESULTS AND DISCUSSION

Primary Structure of Human E2/E3BP—A search of the expressed sequence tag data base of the National Center for Biotechnology Information revealed four clones that encoded protein sequences similar to those reported previously (7, 8, 10) for the amino terminus and an internal region of bovine E2/E3BP. Two of these clones (HS8032 and T77385) were sequenced and found identical to one another except that T77385 is 370 base pairs shorter at its 5′ end. Clone HS8032 has an open reading frame of 1503 base pairs that encodes a protein of 501 amino acid residues with a calculated molecular weight of 54,085 (Fig. 1).

Evidence that this cDNA encodes E2/E3BP is provided by a comparison of the deduced protein sequence with the previously published (7, 8, 10) amino-terminal sequences of bovine heart and kidney E2/E3BP as well as internal sequences obtained with Arg C proteolytic fragments of these proteins (Table I). Although no previous data for the human protein are available in the literature, the almost perfect match (45 of 50 residues) with the bovine protein indicates that the cDNA obtained in this study encodes human E2/E3BP. Based on the amino-terminal sequence of 22 amino acids published previously for bovine heart E2/E3BP (Table I), the mature form of E2/E3BP is assumed to start after a 53-amino acid presequence that has numerous Arg, Ser, and Leu residues arranged in an order consistent with other mitochondrial targeting sequences (21). The assignment of where the mature protein begins is provisional, however, because the mature native human protein has not been subjected to direct amino acid sequencing and there is disagreement in the literature as to whether the amino terminus of the bovine protein is free or blocked (7, 8). If it is assumed that mature E2/E3BP of human starts at the same residue found for the bovine heart protein the human mature protein has 448 amino acids with a calculated molecular weight of 48,040, in reasonably good agreement with the molecular weight of 50,000 estimated by SDS-PAGE (4–6). The sequence that corresponds to the previously determined sequences of the protease Arg C-derived fragments of bovine heart and kidney E2/E3BP follows an Arg-130 in the human protein. Cleavage of the human protein at this residue would generate polypeptides with calculated molecular weights of 14,033 and 34,025, in good agreement with the estimated molecular weights (15,000 and 35,000) by SDS-PAGE of the polypeptides produced by protease Arg C cleavage of the bovine proteins (10, 22).

Size of the Message Encoding E2/E3BP and Chromosomal Loca-
Comparison of previously reported amino-terminal and internal amino acid sequences of bovine heart and kidney E3BP with corresponding DNA-derived sequences of human E3BP

<table>
<thead>
<tr>
<th>Source</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine heart E3BP</td>
<td>ADPKILMPSLTKMEGNIKVE</td>
</tr>
<tr>
<td>Human E3BP</td>
<td>GDIKILMPSLTKMEGNIKVE</td>
</tr>
<tr>
<td>Bovine kidney E3BP</td>
<td>ADPKILMPSLTKMEGNIKVE</td>
</tr>
<tr>
<td>Internal sequences</td>
<td>LSPAARNILEKMQDNLQNX</td>
</tr>
<tr>
<td>Human E3BP</td>
<td>LSPAARNILEKMQDNLQNX</td>
</tr>
<tr>
<td>Bovine kidney E3BP</td>
<td>LSPAARNILEKMQDNLQNX</td>
</tr>
</tbody>
</table>

*N* Sequence reported by Neagle et al. (8) for native bovine heart E3BP with further processing.

*d* Sequence reported by Rahmatullah et al. (7) of a lipoyl-bearing fragment of bovine kidney E3BP produced by limited proteolysis with Arg C and trypsin.

*a* Sequence reported by Sanderson et al. (10) of a non-lipoyl-bearing fragment of bovine heart E3BP produced by limited proteolysis with Arg C.

*b* Sequence reported by Rahmatullah et al. (7) of a non-lipoyl-bearing fragment of bovine kidney E3BP produced by limited proteolysis with Arg C.

The mammalian PDC consists of three single enzyme subcomplexes capable of self-assembly: 1) E1 subcomplex (tetramer of two E1α and two E1β subunits); 2) E2-E3BP subcomplex (consists of 60 copies of E2 and 12 copies of E3BP); and 3) E3 subcomplex (dimer of two identical subunits). This modular organization allows for independent expression of the different single enzyme subcomplexes, which can then be mixed for reconstitution of the multienzyme complex. We took advantage of this fact, generating the catalytically active E1 subcomplex by co-expression of cDNAs for E1α and E1β subunits and independently generating the catalytically active dihydrolipoamide acyltransferase or E2-E3BP subcomplex by co-expression of cDNAs for E2 and E3BP. For comparison purposes, the dihydrolipoamide acyltransferase component lacking E3BP was obtained by expression of the cDNA for E2 alone. The dihydrolipoamide dehydrogenase subcomplex from porcine heart is commercially available in excellent purity, making its expression for the reconstitution studies unnecessary.

The reconstitution of the PDC was achieved by mixing extracts of *E. coli* in which the E1 subcomplex had been expressed with extracts of *E. coli* in which either the E2-E3BP subcomplex or E2 alone subcomplex had been expressed. Reaction mixtures containing expressed E1 alone plus porcine E1β, expressed E2-E3BP alone plus porcine E3, or expressed E2 alone plus porcine E3 did not show PDC activity above that due to the native bacterial enzyme activity present in these extracts (Fig. 2). However, combining extracts containing E1 and E2-E3BP in a reaction cocktail containing porcine E3 reconstituted the mammalian PDC as assayed by more than 3-fold increase in activity above that of the native bacterial enzyme activity (Fig. 2). Combining extracts containing E2 with extracts containing E1 but lacking E3BP in a reaction mixture containing the same amount of porcine E3 resulted in no increase in PDC activity, showing that the cDNA encodes for E3BP and confirming its requirement for the reaction catalyzed by the complex.

To establish whether E3BP physically associates with other
components of the PDC, extracts of E. coli containing the different recombinant proteins were combined to reconstitute subcomplexes with and without E3BP. The E1, E2, E3BP subcomplex, produced by combining extracts containing E1, and E2-E3BP, and the E2 subcomplex, produced by combining extracts containing E2 and E1, were then partially purified by PEG precipitation and gel filtration on Sepharose 4B. Both subcomplexes eluted from the gel filtration column as large macromolecular complexes from the components (data not shown). The isolated E1-E2-E3BP subcomplex added to a reaction mixture containing porcine E3 exhibited a specific enzyme activity of 12 units/mg of protein. This value is lower but still quite respectable relative to previously reported specific activities (18–23 units/mg of protein) for native bovine enzymes (23), particularly since the subcomplex was only partially purified. Incomplete lipoylation of the E2 component could also be a contributing factor. In contrast to the high activity of the E1-E2-E3BP subcomplex, the isolated E1-E2 subcomplex had a much lower specific activity (<0.8 units/mg of protein) when assayed under the same conditions. SDS-PAGE analysis of the respective preparations revealed that the catalytically active subcomplex contains predominant protein bands corresponding to the molecular weights of E2α, E1β, E2, and E2BP (Fig. 3). In contrast, the much less active subcomplex obtained by expression of the E2 cDNA without E2BP cDNA lacks the protein corresponding to E2BP. These findings provide evidence that the E2BP is physically associated with E2 and confirm that it is required for PDC activity.

Comparison of the Amino Acid Sequences of Human E3BP and Human E2—Analysis of the human E3BP amino acid sequence indicates a structure quite similar to human E2, i.e. a segmented domain structure joined by linker regions rich in alanine and proline residues (Fig. 4A). Overall sequence identity is 43% for the two proteins. One difference is that E3BP has one putative lipoyl-bearing domain in its amino-terminal region rather than the two tandemly repeated lipoyl-bearing domains present in E2. Residues 4–83 of E3BP exhibit 44% sequence identity with the first lipoyl-bearing domain and 46% identity with the second lipoyl-bearing domain of E2 (Fig. 4B). Thus, the alignment can be made with either lipoyl-bearing domain, but because of the slightly greater degree of homology, the E3BP sequence has been aligned in Fig. 4 with the second lipoyl-bearing domain of E2. That E3BP should have a lipoyllysine residue in its amino-terminal region is expected from several previous studies demonstrating acetylation of this protein upon incubation of PDC with either radioactive pyruvate or acetyl-CoA (4, 6, 8, 11). Lipoyllysine residues are typically found to occur 43–44 residues from the amino terminus of mammalian E2 proteins (26). A lysine residue is located at assigned position 44 in the deduced sequence of mature E3BP, and this lysine occurs in a typical consensus sequence for a lipoyl-attachment site (branched chain residue-Glu-Ser/Thr-Aep-Lys-Ala-Xaa-branched chain residue) (26) and is, therefore, highly likely to be the site of lipoylation. The existence of a glycine at position 55 is of significance because of recent evidence that a glycine residue situated 11 residues to the carboxyl-terminal side of a lipoylation site is required for lipoylation of E2 by lipoyltransferases (27).

Human E3BP also has an internal region that exhibits considerable sequence identity with the putative E1/E3-binding domain (28) of human E2, (Fig. 4B), a relationship pointed out previously by Sanderson et al. (10) in their analysis of the first 23 amino-terminal residues of an Arg C proteolytic fragment of bovine E3BP. Thirty-eight percent sequence identity occurs in this domain (E2 residues 271 to 303 versus E3BP residues 131–169).
The carboxyl-terminal regions of E2BP and E3 are remarkably similar (Fig. 4B). This is the inner core domain of E2 where acetyl transferase activity as well as the self-assembly sequences are located. These proteins share 50% sequence identity in this region, beginning with residues 221 and 334 of E2BP and E2, respectively, and extending to their carboxyl termini. The carboxyl-terminal region of human E2BP lacks the highly conserved histidine residue found in the active site motif of all dihydroxyoamide acyltransferases. This histidine residue is invariably present in the highly conserved sequence of DHRXXDG in proteins with acyltransferase activity (31–34). The corresponding sequence in human E2BP is DSRXXDD (residues 221 to 226; Fig. 4B). Thus, the active site histidine is replaced by a serine residue, and the carboxyl-terminal aspartate of the motif is replaced by a glycine residue, making it unlikely that this protein possesses acetyltransferase activity.

Based on a comparison of the primary sequences E2 of different species, two domains (residues 428 to 440 and 462 to 483) within the carboxyl-terminal region of E2 have been proposed involved in homo- and hetero-subunit interactions (28). Although not established that these domains are interaction sites between E2 subunits, it is interesting to note that 9 of 13 residues in the first domain and 17 of 22 residues in the second are identical in E2BP and E2. The hinge regions separating the three major segments of E2BP and E2 show the least sequence identity (15%) of any region of these proteins (Fig. 4B). The hinge of E2 that connects lipoyl-bearing domain 2 with the peripheral subunit binding domain is rich in both proline and alanine residues. The corresponding region of E2BP is rich in proline but contains no alanine residues. Collagenase specifically cleaves within this hinge of E2 because it contains an amino acid sequence (PAGP) recognized by this protease. Absence of this sequence from the hinge of E2BP prevents cleavage by collagenase, accounting for the specific removal of lipoyl-bearing domains from E2 by collagenase digestion of the PDC (35). The hinge separating the E1/E3-binding domain from the inner core is rich in proline and alanine residues in both proteins, but this domain is 28 residues longer in E2BP and consequently has many more proline and alanine residues in this region than E2.

Lipoyl-bearing proteins have invariably been found to migrate on SDS-PAGE gels with aberrantly high molecular weights (29, 36), a feature attributed to an extended region of helical structure due to domains rich in alanine and proline residues (37). It is not apparent why this disparity between SDS-PAGE-estimated molecular weight (50,000) and amino acid content-calculated molecular weight (48,040) is not observed for E2BP.

Comparison of the Amino Acid Sequences of Human E2BP and Yeast E2BP—The human E2BP shows significant sequence similarity to the yeast E2BP only in amino-terminal, lipoyl-bearing domains of these proteins (49% identity between residues 4–83) (Fig. 4B). In this region, both proteins have the signature motif of a lipoyl-bearing domain as discussed above in the sequence comparison with E3. Beyond residue 83, however, very little sequence similarity is found for these proteins that have been assigned a similar function in their respective complexes. The yeast protein lacks a well defined proline-alanine-rich domain that would readily identify a hinge region. On the other hand, a short sequence exists between residues 166 and 175 that has significant homology to sequences within the E1/E3-binding domains of human E2 and E2BP. Conservation of this sequence in both the human and the yeast forms of E2BP

FIG. 4. Diagrammatic representation of the structural domains and comparison of the deduced amino acid sequences of human E2, human E2BP, and S. cerevisiae E2BP. Panel A, diagrammatic representation of structural domains. Panel B, comparison of HE2, human E2BP amino acid sequence (24); HE2BP, human E2BP amino acid sequence deduced from DNA in this study; and YE2BP, S. cerevisiae E2BP amino acid sequence (15). Multiple sequence alignment was made according to the MACAW program (25). Conserved amino acids of all three proteins are shown by the black shading and white type. Conserved amino acids of human E2 and human E2BP are shown by dark gray shading and black type. Sites for protease Arg C cleavage of E2BP and collagenase cleavage of E3 are indicated by arrows. Amino acids defining lipoyl-bearing domains, hinges, E1/E3 binding domains, and inner core regions are indicated by double-headed arrows. Lipoylation site lysine residues are indicated by asterisks. Catalytic site histidine residue of human E2 is identified by a # sign.
suggests this motif may be particularly important for E2-binding. However, it is known that E$_3$-deficient PDC from beef heart cannot be reconstituted with yeast E$_3$ (10), a finding that presumably reflects poor binding of yeast E$_3$ to the mammalian E$_3$BP as a consequence of marked differences in sequence and therefore structure of the E$_3$-binding domains of yeast and mammalian E$_3$-binding proteins.

The carboxyl-terminal region of yeast E$_3$BP shows no sequence similarity with either human E$_2$ or E$_3$BP (Fig. 4B). The active site motif DHRXXDG characteristic of dihydrolipoamide acyltransferases is not present in yeast E$_3$BP, and no evidence has been presented for acyltransferase activity with either the native or recombinant proteins (38, 39).

The remarkable differences between yeast E$_3$BP and mammalian E$_3$BP coupled with the remarkable similarity between mammalian E$_2$ and mammalian E$_3$BP raise interesting questions, not the least of which is whether anchoring E$_3$ to E$_2$ is the only function of mammalian E$_3$BP. We are particularly interested in a possible role for this protein in mediating regulation of the activity of the various isoenzymes of pyruvate dehydrogenase kinase that have been discovered recently (19, 40). The availability of the recombinant E$_3$BP and the capability for reconstitution of PDC completely from recombinant proteins should facilitate future studies of the function of protein X.

Acknowledgments—We thank Dr. Byoung J. Song for help with the expression system for E1 and Patricia A. Jenkins for help in preparation of this manuscript.

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Dihydrolipoamide Dehydrogenase-binding Protein of the Human Pyruvate Dehydrogenase Complex: DNA-DERIVED AMINO ACID SEQUENCE, EXPRESSION, AND RECONSTITUTION OF THE PYRUVATE DEHYDROGENASE COMPLEX

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doi: 10.1074/jbc.272.32.19746

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