Vol. 153, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1988 Pages 328-333

MOLECULAR CLONING, SEQUENCE ANALYSIS, AND EXPRESSION OF A HUMAN LIVER cDNA CODING FOR FRUCTOSE-6-P,2-KINASE:FRUCTOSE-2,6-BISPHOSPHATASE

Joseph Algaier and Kosaku Uyeda

Research Service of the Veterans Administration Medical Center and Department of Biochemistry of the University of Texas Southwestern Medical Center, 4500 South Lancaster Rd., Dallas, TX 75216

Received April 4, 1988

A cDNA coding for 378 amino acids from the C-terminus of the human liver bifunctional enzyme, Fructose-6-phosphate,2-kinase:Fructose-2,6-bisphosphatase was isolated, sequenced, and expressed in E. coli K38. The expressed protein, identified by specific immunoassay, showed Fru 2,6-bisphosphatase activity but no Fru 6-P,2-kinase activity, demonstrating directly that the Fru 2,6-bisphosphatase activity resides in the C-terminal region. The Km for Fru 2,6-P_2 was 4.3 μM. Fru 6-P was a noncompetitive inhibitor (Ki = 2.9 μM), and formed a phosphorylated intermediate when incubated with Fru 2,6[2-^{32}P]P_2. The subunit Mr of the enzyme was 36,600, and the active enzyme showed Mr=37,000 by gel filtration.

INTRODUCTION: The synthesis and degradation of fructose-2,6-P_2 are catalyzed by a bifunctional enzyme, Fructose-6-P,2-kinase:Fructose-2,6-bisphosphatase (Fru 6-P + MgATP $\rightleftharpoons$ Fru 2,6-P_2 + MgADP, Fru 2,6-P_2 $\longrightarrow$ Fru 6-P + Pi). Various chemical modification reactions (1,2), proteolysis (3,4), and immuno-reactions (5) have shown that the active sites of the kinase and the phosphatase are distinct, suggesting the existence of two separate domains. The amino acid sequence (6) and the full cDNA sequence for rat liver enzyme (7) were reported recently. We have searched for a cDNA encoding for one of the enzyme activities in order to demonstrate directly such separate domains. In this communication we describe the isolation and DNA sequence analysis of a cDNA which encodes only Fructose-2,6-bisphosphatase of the human liver bifunctional enzyme. In addition, we describe the expression of this enzyme in E. coli, the partial purification, and characterization of the expressed enzyme.

MATERIALS AND METHODS: The human liver λgt11 cDNA library was provided by Dr. William J. Rutter (Univ. of California at San Francisco). The pT7-7 RNA polymerase/promoter plasmid was provided by Dr. Stan Tabor (Harvard Medical School). Antibodies against rat liver Fru 6-P,2-kinase:Fru 2,6-bisphosphatase were raised in a goat as described previously (8). Other materials were purchased from commercial sources.

0006-291X/88 $1.50

The cDNA library was screened with antibodies by the procedure of Huynh et al. (9). Phage DNA from an antibody positive clone was identified, and the Fru 2,6-bisphosphatase DNA was isolated (10). After cloning the Fru 2,6-bisphosphatase DNA into M13 (11) the nucleotide sequence of the DNA was determined by the dideoxynucleotide method (12). The Fru 2,6-bisphosphatase DNA was ligated into the expression vector, pT7-7, and transformed into E. coli K38 that contains pG1-2 (13). The cells, grown in LB medium (10) with kanamycin and ampicillin (50 µg/ml each), were collected by centrifugation, frozen in liquid nitrogen, and stored at -70°C. The cloned Fru 2,6-bisphosphatase was purified to specific activity of 2 milliunits/mg, while control cells expressed no activity. The assay methods for Fru 6-P,2-kinase and Fru 2,6-bisphosphatase were described previously (14). SDS-PAGE was performed on 10% gels according to the procedure of Laemmli (15). After transferring to BioRad Zeta probe (16) the expressed enzyme was visualized by immunoassay (17). Protein concentrations were determined by the Bradford method (18).

RESULTS AND DISCUSSION: Characterization of Fru 2,6-bisphosphatase DNA - The nucleotide sequence of the human liver Fru 2,6-bisphosphatase DNA and its deduced amino acid sequence are presented in Figure 1. Comparison of this amino

```
   1 TTTCTTCCAGACAACATGGAAGCCCTGCAAATCAGGAAGCAGTGCGCCCTGGCAGCCCTG
     F  L  P  D  N  M  E  A  L  Q  I  R  K  Q  C  A  L  A  A  L    20
  61 AAGGATGTTCACAACTATCTCAGCCATGAGGAAGGTCATGTTGCGGTTTTTGATGCAACC
     K  D  V  H  N  Y  L  S  H  E  E  G  H  V  A  V  F  D  A  T    40
 121 AACACTACCAGAGAACGACGGTCACTGATCCTGCAGTTTGCAAAAGAACATGGTTACAAG
     N  T  T  R  E  R  R  S  L  I  L  Q  F  A  K  E  H  G  Y  K    60
 181 GTGTTTTTCATTGAGTCCATTTGTAATGACCCTGGCATAATTGCAGAAAACATCAGGCAA
     V  F  F  I  E  S  I  C  N  D  P  G  I  I  A  E  N  I  R  Q    80
 241 GTGAAACTTGGCAGCCCTGATTATATAGACTGTGACCGGGAAAAGGTTCTGGAAGACTTT
     V  K  L  G  S  P  D  Y  I  D  C  D  R  E  K  V  L  E  D  F   100
 301 CTAAAGAGAATTGAGTGCTATGAGGTCAACTACCAACCCTTGGATGAGGAACTGGACAGC
     L  K  R  I  E  C  Y  E  V  N  Y  Q  P  L  D  E  E  L  D  S   120
 361 CACCTGTCCTACATCAAGATCTTCGACGTGGGCACACGCTACATGGTGAACCGAGTGCAG
     H  L  S  Y  I  K  I  F  D  V  G  T  R  Y  M  V  N  R  V  Q   140
 421 GATCACATCCAGAGCCGCACAGTCTACTACCTCATGAATATCCATGTCACACCTCGCTCC
     D  H  I  Q  S  R  T  V  Y  Y  L  M  N  I  H  V  T  P  R  S   160
 481 ATCTACCTTTGCCGACATGGCGAGAGTGAACTCAACATCAGGGGACGCATCGGAGGTGAC
     I  Y  L  C  R  H  G  E  S  E  L  N  I  R  G  R  I  G  G  D   180
 541 TCTGGCCTCTCAGTTCGCGGCAAGCAGTATGCCTATGCCCTGGCCAACTTCATTCAGTCC
     S  G  L  S  V  R  G  K  Q  Y  A  Y  A  L  A  N  F  I  Q  S   200
 601 CAGGGCATCAGCTCCCTGAAGGTGTGGACCAGTCACATGAAGAGGACCATCCAGACAGCT
     Q  G  I  S  S  L  K  V  W  T  S  H  M  K  R  T  I  Q  T  A   220
 661 GAGGCCCTGGGTGTCCCCTATGAGCAGTGGAAGGCCCTGAATGAGATTGATGCGGGTGTC
     E  A  L  G  V  P  Y  E  Q  W  K  A  L  N  E  I  D  A  G  V   240
 721 TGTGAGGAGATGACCTATGAAGAAATCCAGGAACATTACCCTGAAGAATTTGCACTGCGA
     C  E  E  M  T  Y  E  E  I  Q  E  H  T  P  E  E  F  A  L  R   260
 781 GACCAAGATAAATATCACTACCGCTATCCCAAGGGAGAGTCCTATGAGGATCTGGTTCAG
     D  Q  D  K  Y  H  Y  R  Y  P  K  G  E  S  Y  E  D  L  V  Q   280
 841 CGTCTGGAGCCAGTGATAATGGAGCTAGAACGACAGGAGAATGTACTGGTGATCTGCCAC
     R  L  E  P  V  I  M  E  L  E  R  Q  E  N  V  L  V  I  C  H   300
 901 CAGGCTGTTCATGCGTGCCTCCTGGCCTATTTCCTGGATAAAAGTTCAGATGAGCTTCCA
     Q  A  V  H  A  C  L  L  A  Y  F  L  D  K  S  S  D  E  L  P   320
 961 TATCTCAAGTGCCCTCTGCACACAGTGCTCAAACTCACTCCTGTGGCTTATGGCTGCAAA
     Y  L  K  C  P  L  H  T  V  L  K  L  T  P  V  A  Y  G  C  K   340
1021 GTGGAATCCATCTACCTGAATGTGGAGGCCGTGAACACACACCGGGAGAAGCCTGAGAAT
     V  E  S  I  Y  L  N  V  E  A  V  N  T  H  R  E  K  P  E  N   360
1081 GTGGACATCACCCGGGAACCTGAGGAAGCCCTGGATACTGTACCAGCCCACTACTGAGCC
     V  D  I  T  R  E  P  E  E  A  L  D  T  V  P  A  H  Y end     378
1141 CTTTCCAAGAAGTCAAACTGCCTGTGTCTCTCATCGCCTTCCACCTTTAGGAAATGCTAT
1201 CTTTGCTCTTCTCCTACTCTGCCTTGGCCTCACTGAGGCACCCCACTTCCGGAATT
```

Figure 1. The nucleotide sequence of a cDNA for human liver fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase with the deduced amino acid sequence (single letter code under the middle codon). The numbers on the left indicate the nucleotide base number while the numbers on the right indicate the amino acid number. Underlined amino acids indicate differences between the human and the rat liver enzymes (6,7). The TGA (end) is the stop codon for the coding sequencing beyond which is the non-coding DNA region.

acid sequence to the rat liver enzyme sequences (6,7) showed that 92 amino acids were missing from the N-terminus of the human liver enzyme. This sequence was complete, however, through the DNA encoding C-terminal region and extended 107 nucleotide bases into the non-coding region. Although there were 21 amino acid differences (underlined in Fig. 1), the majority were conservative changes and represented a 5.5% change in the residues from the rat to the human enzyme. This protein would be considered neither highly conserved nor rapidly changing, with a mutation rate similar to other intracellular enzymes such as triosephosphate isomerase (5.3%) or lactate dehydrogenase H (5.3%) (19). This percent change has been correlated with a 10^8 years difference between the two species (19).

Molecular Weight Determination and Subunit Structure - Following SDS-PAGE and identified by specific immunoassay (Fig. 2, lanes 1 and 2), the Fru 2,6-bisphosphatase showed as a doublet with molecular weights of 36,600 and 35,600, where the smaller protein appeared to be a degradation product of the larger. Based on the deduced amino acid sequence, the cloned Fru 2,6-bisphosphatase molecular weight should approximate 44,000. This lower molecular weight indicated that approximately 65 more amino acids (about 157 total) were missing from the cloned Fru 2,6-bisphosphatase compared to the native form. It is unclear, however, if these additional amino acids were lost from the N- or C-terminii. More importantly, this was the first unequivocal evidence that removal of the N-terminal amino acids resulted in loss of the kinase activity and retention of the phosphatase activity. Thus, the N-terminal region of the native enzyme is essential for kinase activity and the Fru 2,6-bisphosphatase activity clearly resides in the C-terminal region. It was reported that thermolysin digestion of the rat liver enzyme yields an active phosphatase with loss of the kinase activity (20). However, in this study the cleavage site was not known. In addition, Sakakibara et al. (21) suggest that the C-terminal region plays a role in maintaining the Fru 6-P,2-kinase activity. They showed that trypsin digestion of the rat liver enzyme to a Mr 50,000 protein leads to the loss of kinase activity with the retention of Fru 2,6-bisphosphatase activity. Furthermore, this

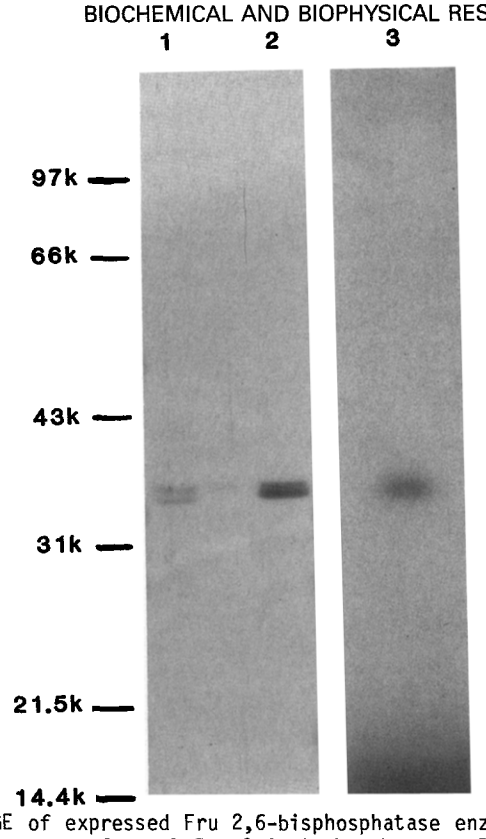

Figure 2. SDS-PAGE of expressed Fru 2,6-bisphosphatase enzyme. Lanes 1 and 2 are the immunostained samples of Fru 2,6-bisphosphatase, DEAE cellulose purified, and Superose 12 purified, respectively. Lane 3 is the autoradiograph of Fru 2,6[2-^{32}P]P_2 labeled Fru 2,6-bisphosphatase.

digested protein contains the blocked N-terminal amino acid, as does the native enzyme suggesting that the trypsin digests the C-terminal region. The rat liver amino acid sequence shows that trypsin cleavage in the C-terminal region at Arg432 (6,7) (our Lys340) would produce a 50,000 protein. This observation suggests to us that the C-terminal region plays a role in maintaining Fru 6-P,2-kinase activity. Thus, if both terminal regions are required for Fru 6-P,2-kinase activity, it may not be possible to isolate a separate Fru 6-P,2-kinase domain.

Since there is no information on the native human liver enzyme, the characteristics of the expressed Fru 2,6-bisphosphatase and the rat liver enzyme were compared. Gel filtration on a Superose 12 column gave Mr of 37,000 and 101,000 for the cloned and rat liver enzymes, respectively (Figure not shown). Thus, under these nondenaturing conditions, the cloned Fru 2,6-bisphosphatase may be active as a monomer, in contrast to the native enzyme which is active as a dimer

(22). These results suggested that the N-terminal region of the intact enzyme may play an important role in the subunit-subunit interaction. The Km for Fru 2,6-P_2 for the cloned Fru 2,6-bisphosphatase was 4.3 μM compared to the rat liver enzyme (1 μM) and the product, Fru 6-P, was a noncompetitive inhibitor of both the expressed and the rat liver (Ki = 2.9 vs 1.5 μM, respectively). In addition, Pi stimulated the activity of both enzymes (maximal activity about 5 mM). When incubated in the presence of Fru 2,6[2-^{32}P]P_2 the labeled Fru 2,6-bisphosphatase migrated identically with the immunoreactive Fru 2,6-bisphosphatase (Fig. 2), confirming the formation of a phospho-intermediate (22). The deduced amino acid sequence revealed that the predicted phosphorylation site (His166, Fig. 1) is conserved, as is 95% of the speculated phosphatase domain (a.a. 159-378) (6).

In summary, we have isolated the human liver Fru 2,6-P,2-kinase:Fru 2,6-bisphosphatase cDNA and expressed it in E. coli. The expressed, enzyme which lacked 92 N-terminal amino acids, retained Fru 2,6-bisphosphatase activity but lacked Fru 6-P,2-kinase activity. Thus, we unequivocally showed for the first time that Fru 2,6-bisphosphatase activity resided in the C-terminal region. The kinetic parameters of the expressed human liver Fru 2,6-bisphosphatase were similar to the native rat liver enzyme, but the former enzyme appears to be active as a monomer while the latter enzyme is active as a dimer. This study also disclosed that the N-terminal region of the native enzyme may be involved in protein-protein subunit interaction as well as being necessary for the Fru 6-P,2-kinase activity. In addition, comparison of an earlier report (21) with the recently published rat liver amino acid sequence (6,7) shows that the C-terminal tail is also necessary for Fru 6-P,2-kinase activity. Availability of this expressed enzyme will allow a more in-depth examination of the Fru 2,6-bisphosphatase active site.

ACKNOWLEDGMENT: This work was supported by the Veterans Administration and by United States Public Health Service Grant No. AM16194. We thank Dr. William J. Rutter (Univ. of California-San Francisco) for providing human liver λgt11 cDNA library and Dr. Stan Tabor for pT7-7 RNA polymerase/promoter plasmid. We also thank Barbara Beard for her skillful technical assistance.

REFERENCES:

1. Kitajima, S., Sakakibara, R., and Uyeda, K. (1984) J. Biol. Chem. 259, 6896-6903.
2. Kitajima, S., Thomas, H., and Uyeda, K. (1985) J. Biol. Chem. 260, 13995-14002.
3. Sakakibara, R., Kitajima, S., and Uyeda, K. (1984) J. Biol. Chem. 259, 8366-8371.
4. El-Maghrabi, M.R., Pate, T.M., Murry, K.J., and Pilkis, S.J. (1984) J. Biol. Chem. 259, 13096-13103.
5. Van Schaftingen, E., Coulie, P.G., Van Snick, J., and Hers, H.G. (1986) Eur. J. Biochem. 159, 367-373.
6. Lively, M.O., El-Maghrabi, M.R., Pilkis, J., D'Anyelo, G., Colosia, A.D., Ciavola, J., Fraser, B.A., and Pilkis, S.J. (1988) J. Biol. Chem. 263, 839-849.
7. Darville, M.I., Crepin, K.M., Vandekerckhoue, J., Van Damme, J., Octave, J.N., Rider, M.H., Marchand, M.J., Hue, L., and Rousseau, G.G. (1987) FEBS Lett. 224, 317-321.
8. Thomas, H., Taniyama, M., Kitajima, S., and Uyeda, K. (1986) Fed. Proc. 45, 1013.
9. Huynh, T.V., Young, R.A., and Davis, R.W. (1985) in: DNA Cloning, vol. 1 (Glover, D.M., ed.), pp. 49-78. IRL Press, Washington, DC.
10. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory), Cold Spring Harbor, NY.
11. M13 Cloning/Dideoxy Sequencing Manual (Bethesda Research Laboratories), Gaithersburg, MD.
12. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
13. Tabor, S. and Richardson, C. (1985) Proc. Natl. Acad. Sci. USA 82, 1074-1078.
14. Sakakibara, R., Kitajima, S., and Uyeda, K. (1984) J. Biol. Chem. 259, 41-46.
15. Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.
16. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
17. Harpold, M.M., Dobner, P.R., Evans, R.M., and Bancroft, F.L. (1981) Nucl. Acids Res. 5, 2309-2315.
18. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
19. Wilson, A.C., Carlson, S.S., and White, T.J. (1977) Ann. Rev. Biochem. 46, 573-639.
20. El-Maghrabi, M.R., Pate, T.M., Murray, K., and Pilkis, S.J. (1984) J. Biol. Chem. 259, 13096-13103.
21. Sakakibara, R., Tanaka, T., Uyeda, K., Richards, E.G., Thomas, H., Kangawa, K., and Matsuo, H. (1985) Biochemistry 24, 6818-6824.
22. Pilkis, S.J., Walderhaug, M., Murray, K., Beth, A., Venkataramu, S.D., Pilkis, J., and El-Maghrabi, M.R. (1983) J. Biol. Chem. 258, 6135-6141.