J. Biochem. 122, 122-128 (1997)

Characterization of a Human Placental Fructose-6-Phosphate, 2-Kinase/Fructose-2,6-Bisphosphatase[1]

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Received for publication, February 13, 1997

A full-length cDNA, which encodes a human placental fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase, was constructed and expressed in *Escherichia coli*. The expressed protein, purified to homogeneity, showed a molecular weight of 58,000 by gel electrophoresis under denaturing conditions, compared to the deduced molecular weight of 59,410. The N-terminal sequence of 15 amino acids coincided with that of the deduced sequence. The active enzyme was a dimer as judged by molecular sieve filtration. The expressed enzyme was bifunctional with V_{max} values of 142 and 0.2 milliunits/mg for the kinase and phosphatase activities, respectively. The phosphatase activity was extremely low, because one phosphatase active site residue was mutated, and consequently the kinase/phosphatase ratio was the highest among the known isozymes. Furthermore, the enzyme was phosphorylated by cAMP-dependent protein kinase, protein kinase C and also by [2-^{32}P]fructose-2,6-bisphosphate. Phosphorylation by cAMP-dependent protein kinase and protein kinase C increased the maximal Fru-6-P,2-kinase activities by 1.8- and 1.1-fold, respectively. These results suggested that placental fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase is important in maintaining and regulating a relatively high rate of glycolysis in placenta.

Key words: bifunctional enzyme, fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase, human placenta, isozyme, phosphorylation.

The bifunctional enzyme fructose-6-phosphate,2-kinase [EC 2.7.1.105]/fructose-2,6-bisphosphatase [EC 3.1.3.46] (Fru-6-P,2-kinase/Fru-2,6-BPase) catalyzes the synthesis (Fru-6-P + ATP $\rightleftarrows$ Fru-2,6-P_2 + ADP) and hydrolysis (Fru-2,6-P_2 + $H_2O \rightarrow$ Fru-6-P + P_i) of fructose-2,6-bisphosphate (Fru-2,6-P_2), which is the most powerful activator of phosphofructokinase (*1*), a key regulatory enzyme of glycolysis.

Several isozymes of the bifunctional enzyme have been identified in mammalian tissues. They differ in physicochemical and immunological properties and in response to phosphorylation by protein kinases. According to the tissue distribution, they are classified as liver, skeletal muscle, heart, and testis isozymes. Their amino acid sequences have been determined from the corresponding cDNAs (*2-6*). All Fru-6-P,2-kinase/Fru-2,6-BPase isozymes are homodimers with subunit molecular weights of about 55,000, and each subunit contains two separate catalytic domains; the N-terminal and C-terminal halves of the subunit for the kinase and phosphatase reactions, respectively. The bovine brain isozyme is also reported to be a homodimer, with a subunit molecular weight twice (120,000) (*7*) those of the above isozymes.

The cellular concentration of Fru-2,6-P_2 is determined by the relative activities of the kinase and the phosphatase. The most important regulatory mechanism of these opposing activities is *via* phosphorylation and dephosphorylation of the enzymes. The liver isozyme is phosphorylated by cAMP-dependent protein kinase, which results in inhibition of the kinase and activation of the phosphatase (*8-11*). The skeletal muscle isozyme is not phosphorylated by cAMP-dependent protein kinase, since the phosphorylation site target Ser-32 of the liver isozyme is replaced by Ala in the muscle isozyme (*12*). In contrast to the liver isozyme, the heart isozyme is phosphorylated by both cAMP-dependent protein kinase and protein kinase C, resulting in activation rather than inhibition of the kinase activity (*13, 14*). On the other hand, Sakata *et al.* (*6*) reported that the testis isozyme is phosphorylated by protein kinase C, but not by cAMP-dependent protein kinase.

Previously, we cloned a cDNA encoding a Fru-6-P,2-kinase/Fru-2,6-BPase from human first trimester placenta (*15*). Based on its deduced amino acid sequence, this protein is a novel isozyme of Fru-6-P,2-kinase/Fru-2,6-BPase, which could be a placental-form isozyme. In this report we describe the expression and characterization of human placental Fru-6-P,2-kinase/Fru-2,6-BPase.

[1] This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: Fru-2,6-P_2, fructose-2,6-bisphosphate; Fru-6-P, fructose-6-phosphate; Fru-6-P,2-kinase/Fru-2,6-BPase, fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase; HP, human placental Fru-6-P,2-kinase/Fru-2,6-BPase cDNA; M_r, molecular weight, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Materials—Restriction enzymes were purchased from New England Biolabs, DNA polymerase I large fragment from Nippon Gene, and T4 DNA ligase from Takara. The pT7-7 RNA polymerase/promoter plasmid (*16*) was a gift of Dr. Stan Tabor (Harvard Medical School). Catalytic subunit of cAMP-dependent protein kinase, protein kinase C, phosphatidylserine, diolein, and Fru-2,6-P_2 were purchased from Sigma. [^{32}P]ATP was obtained from Amersham. [2-^{32}P]Fru-2,6-P_2 was prepared as described in Ref. *11* using expressed rat testis Fru-6-P,2-kinase/Fru-2,6-BPase (*17*). All other chemicals were of reagent grade, obtained from commercial sources.

Construction of the pT7-7/HP2K Expression Plasmid—In a previous study (*15*), we cloned cDNA fragments 2K-3 (nucleotides 705 to 1756 of HP) and AP-4 (nucleotides 1 to 733 of HP) for a human placental Fru-6-P,2-kinase/Fru-2,6-BPase cDNA (designated as HP). To synthesize an expression vector containing the full-length cDNA encoding the placental Fru-6-P,2-kinase/Fru-2,6-BPase, we used these two cDNA fragments of HP and a newly synthesized DNA fragment FP-3 (nucleotides 178 to 1149 of HP) in a reverse transcription-polymerase chain reaction. The procedure for the construction of the expression vector was as follows (Fig. 1). To synthesize the full-length cDNA encoding the placental Fru-6-P,2-kinase/Fru-2,6-BPase, pBluescript-containing fragment AP-4 [pBS KS(+)/AP-4] was digested with *Nco*I and *Xho*I. Insertion of the *Nco*I-*Xho*I fragment prepared from pBS KS(+)/FP-3 (pBluescript-containing fragment FP-3) into the *Nco*I-*Xho*I-digested pBS KS(+)/AP-4 yielded a construct, pBS KS(+)/FA-6. pBS SK(+)/2K-3 and pBS KS(+)/FA-6 were digested with *Stu*I and *Eco*RI. The *Eco*RI/*Stu*I fragment of pBS KS(+)/FA-6 was isolated and introduced into the *Eco*RI/*Stu*I-digested pBS SK(+)/2K-3, and the construct was introduced in the *Sal*I site of pBluescript KS(+) [the full-length cDNA of the human placental Fru-6-P,2-kinase/Fru-2,6-BPase, pBS KS(+)/HP2K]. pBS KS(+)/HP2K was double-digested with *Bam*HI/*Bgl*II and then ligated with synthetic oligoDNA having *Bam*HI-*Bgl*II cohesive ends and an internal *Nde*I site (5′-GATCCTCAT-ATGCCGTTGGAACTGACGCAGAGCCGAGTGCAGAA-3′), which was digested with *Nde*I/*Sal*I to match the reading frame of the bacteriophage T7 direct expression system (*16*). Insertion of this fragment into the *Nde*I-*Sal*I-digested pT7-7 yielded a construct, pT7-7/HP2K, that contained

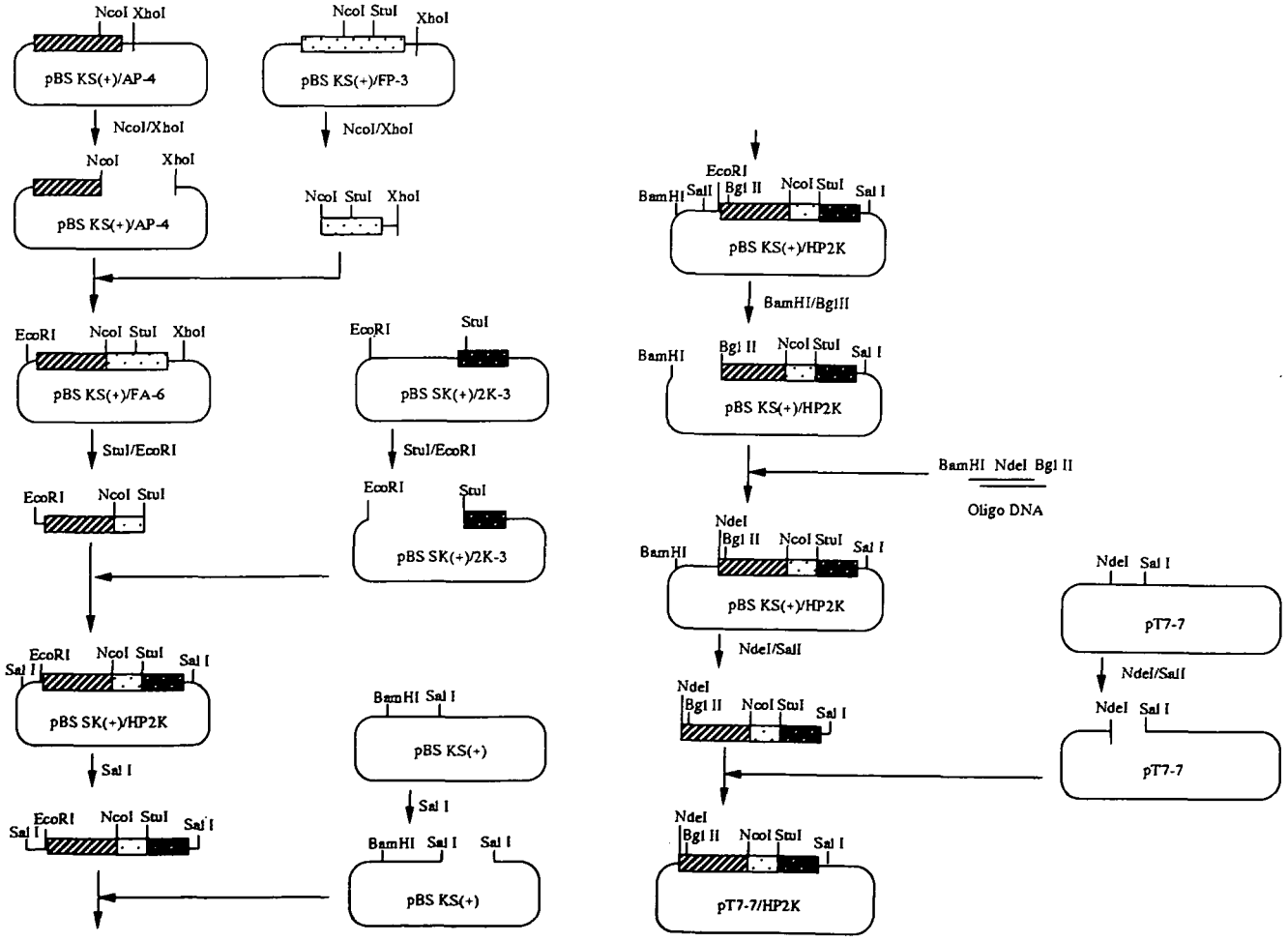

Fig. 1. **Schematic description of the construction of an expression vector, pT7-7/HP2K, containing the full-length placental Fru-6-P,2-kinase/Fru-2,6-BPase cDNA.**

the full length human placental Fru-6-P,2-kinase/Fru-2,6-BPase cDNA attached directly to the translation initiation codon of pT7-7. The constructed cDNA was validated by dideoxynucleotide sequencing and restriction analysis.

Expression of the Human Placental Fru-6-P,2-Kinase/Fru-2,6-BPase in E. coli—The expression plasmid was transformed into *Escherichia coli* BL21(DE3). The culture was grown at 32°C until the absorbance at 595 nm of the culture reached 0.5 in 2% Tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, 50 mM potassium phosphate, pH 8.0, and 50 μg/ml ampicillin. After addition of 30 μM isopropyl-β-D-thiogalactoside, the culture was incubated at 22°C for 44–48 h.

Purification of Expressed Human Placental Fru-6-P,2-Kinase/Fru-2,6-BPase—A 2-liter culture of *E. coli* BL21(DE3) containing pT7-7/HP2K was harvested by centrifugation. The pellet was suspended in two volumes (v/w) of 50 mM Tris-phosphate buffer (pH 8.0), 10 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.02% phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 10 U/ml of aprotinin (Buffer A). After addition of lysozyme (1 mg/ml), the suspension was kept on ice for 30 min. The suspension was subjected to sonication for 1 min, and the cell lysate was centrifuged for 30 min at 18,000×g. Protamine-sulfate was added to the lysate to bring its concentration to 0.38% and centrifuged for 30 min at 18,000×g. Polyethylene glycol (M_r=6,000) was added to the supernatant solution to bring its concentration to 10% and after 1 h the precipitate was collected by centrifugation. The precipitate was dissolved in 10 ml of Buffer A, and the enzyme was adsorbed on a DE52 column (3×7 cm) which had been equilibrated with 50 mM Tris-phosphate, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreitol, 1% polyethylene glycol (M_r=300), and 1% glycerol (Buffer B). The column was washed with the same buffer until the absorbance at 280 nm was less than 0.1. The enzyme was eluted from the column with a linear gradient consisting of 200 ml each of 0 to 0.3 M potassium phosphate, pH 8.0, in Buffer B. The enzyme fractions were pooled and concentrated to 1 ml with an Amicon concentrator equipped with a YM-10 membrane. The concentrated enzyme was applied on a Sephadex G-100 column (1.5×90 cm) which had been equilibrated with Buffer B. The major enzyme fractions were pooled, concentrated, and stored at −80°C.

Assay Method for Fru-6-P,2-Kinase—The activity of Fru-6-P,2-kinase was assayed as described previously (*11*) with slight modifications. The reaction mixture contained, in a final volume of 0.1 ml, 100 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 0.1 mM EDTA, 5 mM ATP, 1 mM Fru-6-P, 5 mM potassium phosphate, 10 mM $MgCl_2$, and an appropriate amount of enzyme. The mixture was incubated at 30°C, and at intervals, aliquots (10 μl) were transferred to 90 μl of 50 mM Tris-HCl, pH 8.0, and the diluted solution was heated for 1 min at 90°C to stop the reaction. Suitable aliquots of the heated reaction mixture were then assayed for Fru-2,6-P_2 as described by Uyeda *et al.* (*18*). One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru-2,6-P_2 per min under these conditions.

Assay Method for Fru-2,6-BPase—The activity of Fru-2,6-BPase was assayed as described previously (*11*). The reaction mixture contained, in a final volume of 0.1 ml, 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM NADP, 1 unit of desalted glucose-6-P dehydrogenase, 0.4 unit of phosphoglucose isomerase, and 10 μM [^{32}P-2]Fru-2,6-P_2 (2×10^8 cpm/mmol). The reaction was initiated by the addition of the enzyme, and the reaction mixture was incubated at 30°C. At intervals, aliquots were removed and transferred into 100 μl of 0.1 N NaOH, and the solution was heated at 100°C for 2 min. H_2O (1 ml) was added to the heated reaction mixture and the whole was adsorbed on a Dowex-1×8-Cl^- column (bed volume of 0.5 ml) which had been equilibrated with 20 mM NH_4OH. The column was washed with 1 ml of 20 mM NH_4OH. [^{32}P]Phosphate was then eluted with 4 ml of 0.15 M NaCl in 20 mM NH_4OH, and a portion (1 ml) of the eluate was diluted in 6 ml of Ready Gel (Beckman) and counted in a scintillation counter. One unit of the activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of phosphate per min under those conditions.

Phosphoenzyme Assay—The reaction mixture contained, in a final volume of 0.1 ml, 100 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 10 mM $MgCl_2$, 0.2 mM EDTA, 5 mM potassium phosphate (pH 7.5), 100 μM [2-^{32}P]Fru-2,6-P_2 (200 cpm/pmol), and 1.17 μM expressed human placental Fru-6-P,2-kinase/Fru-2,6-BPase or 1.2 μM expressed rat testis Fru-6-P,2-kinase/Fru-2,6-BPase. The reaction mixture was incubated at 30°C. At the indicated time intervals, 10 μl aliquots were removed for SDS-PAGE. The dried gel was subjected to image analysis using an image analyzer (BAS1000), and the radioactivity of [^{32}P]phosphate incorporated into the enzyme band was counted.

Phosphorylation by Protein Kinases—For phosphorylation by cAMP-dependent protein kinase, the reaction mixture contained, in a final volume of 0.1 ml, 0.14 mg/ml of human placental Fru-6-P,2-kinase/Fru-2,6-BPase, 50 mM Tris-phosphate (pH 7.5), 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 10 mM $MgCl_2$, and 0.2 mM [^{32}P]ATP (850 cpm/pmol), and the reaction was initiated with 0.5 unit of catalytic subunit of cAMP-dependent protein kinase. For phosphorylation by protein kinase C, the reaction mixture contained, in a final volume of 0.1 ml, 0.14 mg/ml of human placental Fru-6-P,2-kinase/Fru-2,6-BPase, 50 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 5 mM $MgCl_2$, 0.2 mM [^{32}P]ATP (850 cpm/pmol), 1.5 mM $CaCl_2$, 100 μg/ml phosphatidylserine, and 2 μg/ml diolein, and the reaction was initiated with 0.5 unit of protein kinase C. The reaction mixture was incubated at 30°C. At the indicated time intervals, 10 μl aliquots were removed for SDS-PAGE. The dried gel was subjected to image analysis using an image analyzer (BAS1000), then the radioactivity of [^{32}P]phosphate incorporated into the enzyme band was counted.

Other Methods—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (*19*) using 12% gel. The gels were stained using Quick CBB (Wako Chemicals). Protein concentration was determined by the Bradford method (*20*) using bovine serum albumin as a standard.

RESULTS

Purification of Expressed Human Placental Fru-6-P,2-Kinase/Fru-2,6-BPase—A typical example of this purification procedure is summarized in Table I. The purified

enzyme was homogeneous as judged by SDS-PAGE (Fig. 2). In some preparations, minor protein bands with lower M_r were detected below the major enzyme protein band on SDS-PAGE. The amount of lower M_r proteins varied depending on the preparation, and in most preparations these proteins were absent. These proteins appeared to be proteolysis products, since they were detected after storage of the purified enzyme at 4°C for several days. These lower M_r proteins could be removed by re-chromatography on DE52, as described above.

Physicochemical Properties of the Expressed Enzyme—The expressed human placental Fru-6-P,2-kinase/Fru-2,6-BPase should contain 519 amino acids, and the calculated M_r of the subunit was 59,410 (*15*). The SDS-PAGE showed that the expressed human placental enzyme had the subunit M_r of 58,000 (Fig. 2). Based on the elution position of the enzyme from a Sephadex G-100 column, the M_r of the active enzyme was estimated to be 120,000, indicating that the enzyme was a dimer. Amino-terminal amino acid

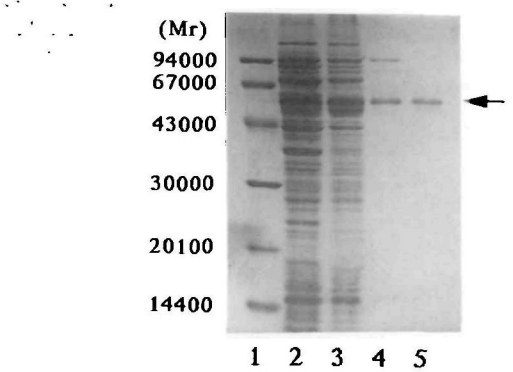

Fig. 2. **SDS-PAGE of various fractions of Fru-6-P,2-kinase/Fru-2,6-BPase.** Aliquots of the fractions were subjected to SDS-PAGE. Lane 1, marker proteins (from top): phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), α-lactalbumin (14,400); lane 2, crude extract (10 μg of protein); lane 3, polyethylene glycol precipitate (10 μg of protein); lane 4, DE-52 eluate (1 μg of protein); and lane 5, Sephadex G-100 eluate (1 μg of protein).

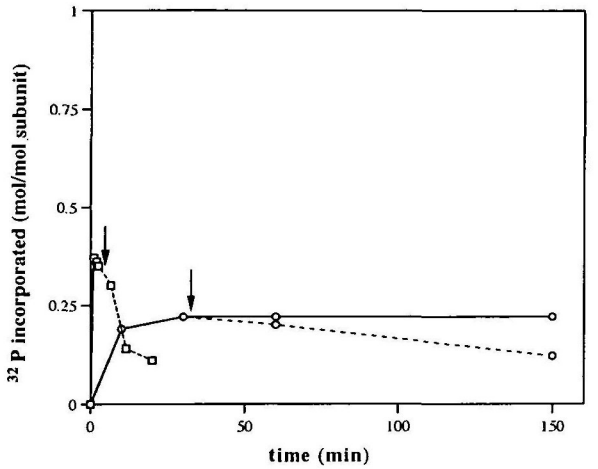

Fig. 3. **Phosphorylation of human placental Fru-6-P,2-kinase/Fru-2,6-BPase by Fru-2,6-P_2.** The phosphorylation of expressed human placental (○) and expressed rat testis Fru-6-P,2-kinase/Fru-2,6-BPase (□) by 100 μM [2-^{32}P]Fru-2,6-P_2 was performed as described under "MATERIALS AND METHODS." When the incorporation of ^{32}P reached the steady-state level, non-labeled Fru-2,6-P_2 (10 mM) was added to the reaction mixture as indicated by arrows. The changes of the amount of ^{32}P incorporated into enzymes after the addition of non-labeled Fru-2,6-P_2 are shown by dotted lines. At the indicated time intervals, aliquots were removed for SDS-PAGE, and the radioactivity of [^{32}P]phosphate incorporated into the enzyme band was counted.

TABLE I. ***Purification of expressed human placental Fru-6-P,2-kinase/Fru-2,6-BPase.***

	Volume (ml)	Fru-6-P,2-kinase				Fru-2,6-BPase[a]	Kinase/Pase[b]
		Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Recovery (%)	Total activity (mU)	
Crude extract	12.5	244	688	2.8	100	ND[c]	–
Polyethylene glycol	7	87	600	6.9	87	ND	–
DE 52	40	3.8	418	110	61	0.30	1,393
Sephadex G-100	15	2.7	383	142	56	0.27	1,419

[a]Fru-2,6-BPase activity was determined in the presence of 10 μM Fru-2,6-P_2. [b]Kinase/Pase, Fru-6-P,2-kinase:Fru-2,6-BPase. [c]ND, not detectable.

TABLE II. **Comparison of the kinetic properties of various Fru-6-P,2-kinase/Fru-2,6-BPase isozymes.** The values for liver, heart, skeletal muscle, testis, and brain enzymes were taken from Refs. *11*, *22*, *12*, *6*, and *7*, respectively. Fru-6-P,2-kinase activity was determined as described under "MATERIALS AND METHODS" except that for the determination of K_m for Fru-6-P, ATP concentration was kept at 5 mM and Fru-6-P was varied, and for K_m determination for ATP the Fru-6-P was kept at 5 mM and ATP was varied. Fru-2,6-BPase activity was determined as described under "MATERIALS AND METHODS" except that the Fru-2,6-P_2 concentration was varied from 1 to 160 μM.

	Placenta human	Liver rat	Heart bovine	Skeletal muscle rat	Testis rat	Brain bovine
Kinase						
V_{max} (mU/mg)	142	57	61	66	90	90
K_m (μM)						
Fru-6-P	32	16	74	56	85	27
ATP	220	250	260	48	270	55
Bisphosphatase						
V_{max} (mU/mg)	0.2	45	10	154	22	29
K_m (μM)	130	0.5	40	0.4	21	70
Kinase/bisphosphatase	710	1.3	6.1	0.4	4.1	3.1

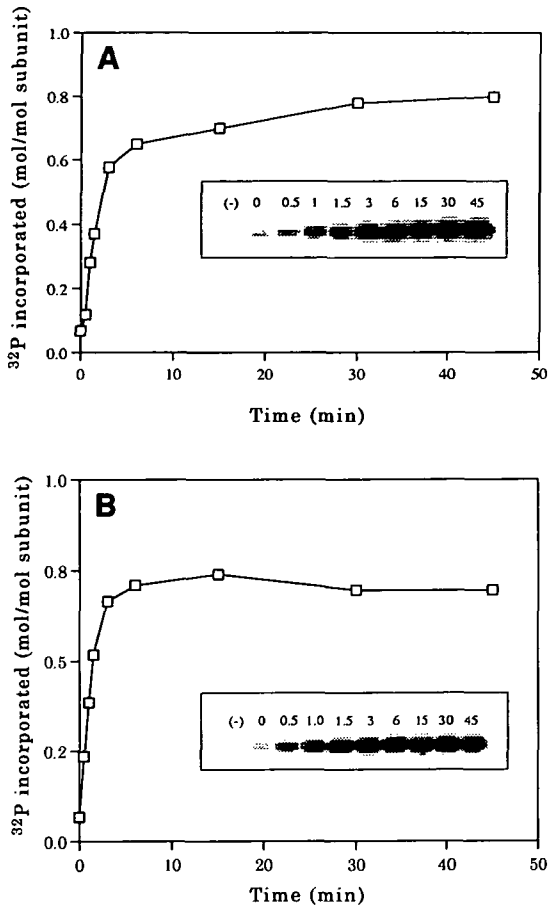

Fig. 4. **Phosphorylation of human placental Fru-6-P,2-kinase/Fru-2,6-BPase by cAMP-dependent protein kinase (A) and by protein kinase C (B).** The reaction mixture and the conditions were as described under "MATERIALS AND METHODS." At the indicated time intervals, 10 μl aliquots were removed for SDS-PAGE, and the radioactivity of [^{32}P]phosphate incorporated into the enzyme band was counted. The insert shows the image of the radioactive band in the gel obtained with an image analyzer (BAS1000).

sequence analysis (15 cycles) of the expressed enzyme was consistent with that deduced from the cDNA sequence (PLELTQSRVQKIWVP).

Kinetic Properties of the Expressed Enzyme—Table II compares kinetic constants of the expressed human placental enzyme with those of the rat liver (*11*), rat skeletal muscle (*12*), rat testis (*6*), bovine heart (*22*), and bovine brain (*7*) enzymes. The expressed human placental Fru-6-P,2-kinase had the highest kinase activity among these kinases, but interestingly, its Fru-2,6-BPase activity was the lowest. Thus the kinase/bisphosphatase ratio was over 700 compared to the values of 0.4-6.1 for the other isozymes.

Phosphorylation of Fru-2,6-BPase with Fru-2,6-P_2—The Fru-2,6-BPase domain of Fru-6-P,2-kinase/Fru-2,6-BPase incorporates radioactive [^{32}P]phosphate from the substrate, [2-^{32}P]Fru-2,6-P_2, in a ping-pong reaction (*21*), which is called enzyme-phosphate intermediate formation (E-P formation). When the expressed human placental enzyme was incubated with [2-^{32}P]Fru-2,6-P_2, the subunit band was labeled dependent on the time of incubation. The

TABLE III. **Effect of phosphorylation on Fru-6-P,2-kinase activity of human placental Fru-6-P,2-kinase/Fru-2,6-BPase.** Phosphorylations by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) were carried out as described under "MATERIALS AND METHODS" using 0.1 mg/ml of expressed human placental Fru-6-P,2-kinase/Fru-2,6-BPase. The maximal Fru-6-P,2-kinase activity was measured before (native) and after phosphorylation. ± indicates the average of four determinations.

Treatment	Fru-6-P,2-kinase	
	Activity (mU/ml)	%
Native	11.6±0.8	100
PKA	20.5±1.2	177
PKC	12.8±0.7	110

E-P formation reached a steady-state level, and then the liberation of [^{32}P] (E-P breakdown) was observed after addition of excess non-labeled Fru-2,6-P_2 (Fig. 3), indicating that expressed human placental enzyme hydrolyzes Fru-2,6-P_2 through E-P formation. However, the apparent rates of E-P formation and breakdown were significantly slower than those of the expressed rat testis enzyme.

Phosphorylation by Protein Kinases—When the expressed human placental Fru-6-P,2-kinase/Fru-2,6-BPase was incubated with the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP, 0.8 mol of phosphate/mol subunit was incorporated (Fig. 4A). This phosphorylation site of human placental enzyme is expected to be Ser-460 (-RRNS-), based on the consensus amino acid sequence of the phosphorylation site of cAMP-dependent protein kinase. The protein kinase C also phosphorylated human placental Fru-6-P,2-kinase/Fru-2,6-BPase, and 0.8 mol of phosphate/mol of subunit was incorporated (Fig. 4B).

Effect of Phosphorylation on Kinase Activity of Human Placental Fru-6-P,2-Kinase/Fru-2,6-BPase—As shown in Table III, phosphorylation of human placental Fru-6-P,2-kinase/Fru-2,6-BPase by cAMP-dependent protein kinase resulted in increased maximal Fru-6-P,2-kinase activity (177%) compared to the native enzyme. Phosphorylation by protein kinase C also increased the Fru-6-P,2-kinase activity, but only slightly (110%). The K_m values for ATP and Fru-6-P of the enzyme were little affected by phosphorylation with either protein kinase. The Fru-2,6-BPase activity was not affected by the treatments with these protein kinases.

DISCUSSION

Our previous results of cloning and characterization of the cDNA (HP) from human placenta, based on the deduced amino acid sequence and the size of mRNA, showed that this cDNA codes a novel isozyme of Fru-6-P,2-kinase/Fru-2,6-BPase (*15*). The purpose of the present study was to characterize this isozyme of Fru-6-P,2-kinase/Fru-2,6-BPase, which is a placenta-specific isozyme. The expressed placental Fru-6-P,2-kinase/Fru-2,6-BPase was purified to homogeneity by polyethylene glycol precipitation, DEAE-cellulose chromatography, and gel-filtration. The expressed protein was validated by N-terminal amino acid sequencing. The enzyme is a homodimer with subunit M_r of 58,000, and this agrees with the calculated value based on the deduced amino acid sequence.

Various kinetic constants of the expressed placental

enzyme were different from those of other major isozymes of liver, skeletal muscle, heart, testis, and brain (Table II). In particular, the placental isozyme showed extremely low phosphatase activity compared with other isozymes. The maximal velocity of Fru-2,6-BPase reaction is dependent on the rates of the formation of E-P intermediate and its breakdown (*23, 24*). In the Fru-2,6-BPase reaction, the formation and breakdown of the E-P intermediate proceed within several seconds and minutes, respectively. However, the apparent rates of both E-P formation and its breakdown in the phosphatase reaction of human placental Fru-2,6-BPase (Fig. 3) were very slow, which accounts for its low phosphatase activity. The crystal structure of the rat testis isozyme shows that His-256, Arg-255, Arg-305, Glu-325, and His-390 are located in the catalytic center of Fru-2,6-BPase, and they are important in the Fru-2,6-BPase reaction (*25*). These residues are highly conserved among various isozymes. The corresponding residues, namely His-253, Arg-252, Glu-322, and His-387, of the human placental isozyme, were also conserved, but the residue corresponding to Arg-305 of the testis isozyme was substituted to Ser-302 in the placental enzyme (*15*). Thus, the extremely low activity of human placental Fru-2,6-BPase is most likely caused by this change of Arg to Ser, because it has been reported that the maximal activity of Fru-2,6-BPase of a mutated rat liver isozyme (Arg-307, corresponding to Arg-305 of testis isozyme, to Ala) is 700-fold lower than that of the wild-type enzyme (*26*).

The activity ratio of kinase/phosphatase was the highest among the reported isozymes. The high kinase/phosphatase ratio suggests that the placental Fru-6-P,2-kinase/Fru-2,6-BPase maintains a high Fru-2,6-P_2 level and as a result, PFK of placenta may be kept active. These results explain how the placenta exhibits a relatively high rate of glycolysis for high energy production and for the supply of substrates for the development and maintenance of the fetus.

One of the important regulatory mechanisms of the liver and heart isozymes is phosphorylation and dephosphorylation. The deduced amino acid sequence of placental Fru-6-P,2-kinase/Fru-2,6-BPase showed the consensus sequences of the phosphorylation sites for cAMP-dependent protein kinase (Ser-460) and protein kinase C (Ser-51, Thr-128, Ser-271, Thr-440, Thr-470, and Ser-516) (*15*). Indeed, the placental Fru-6-P,2-kinase/Fru-2,6-BPase was phosphorylated by cAMP-dependent protein kinase, like the liver and heart isozymes. Approximately 1 mol of [^{32}P]-phosphate was incorporated into 1 mol of subunit, presumably at Ser-460. The placental Fru-6-P,2-kinase/Fru-2,6-BPase is similar to the heart isozyme and is phosphorylated stoichiometrically by protein kinase C. Which residue among six putative phosphorylation sites is phosphorylated by protein kinase C needs to be elucidated. The maximal kinase activity of the liver enzyme is decreased upon phosphorylation by cAMP-dependent protein kinase (*11*). In contrast, the maximal activity of the heart enzyme is little affected by phosphorylation with cAMP-dependent protein kinase, whereas phosphorylation by protein kinase C increases the maximal activity by 1.5-fold (*14*). On the other hand, in the case of human placental Fru-6-P,2-kinase/Fru-2,6-BPase, the maximal activity of Fru-6-P,2-kinase was significantly (1.8-fold) and slightly (1.1-fold) increased upon phosphorylation by cAMP-dependent protein kinase and by protein kinase C, respectively (Table III). These results and the evidence of the high kinase/phosphatase ratio of placental Fru-6-P,2-kinase/Fru-2,6-BPase as described above suggest that the glycolytic rate of placenta may be regulated at a higher level *via* the cAMP-dependent protein kinase and/or protein kinase C systems in response to some signal(s). It would be of great interest to identify the signal(s) causing phosphorylation and dephosphorylation of human placental Fru-6-P,2-kinase/Fru-2,6-BPase *in vivo*.

REFERENCES

1. Uyeda, K., Furuya, E., Richard, C.S., and Yokoyama, M. (1982) Fructose-2,6P_2, chemistry and biological function. *Mol. Cell. Biochem.* **48**, 97-120
2. Darville, M.I., Crepin, K.M., Vandekerckhove, J., Van Damme, J., Octave, J.-N., Rider, M.H., Marchand, M.J., Hue, L., and Rousseau, G.G. (1987) Complete nucleotide sequence coding for rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase derived from a cDNA clone. *FEBS Lett.* **224**, 317-321
3. Colosia, A., Lively, M., El-Maghrabi, M.R., and Pilkis, S.J. (1987) Isolation of a cDNA clone for rat liver 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase. *Biochem. Biophys. Res. Commun.* **143**, 1092-1098
4. Crepin, K.M., Darville, M.I., Michel, A., Hue, L., and Rousseau, G.G. (1989) Cloning and expression in *Escherichia coli* of a rat hepatoma cell cDNA coding for 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Biochem. J.* **264**, 151-160
5. Sakata, J. and Uyeda, K. (1990) Bovine heart fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase: Complete amino acid sequence and localization of phosphorylation sites. *Proc. Natl. Acad. Sci. USA* **87**, 4951-4955
6. Sakata, J., Abe, Y., and Uyeda, K. (1991) Molecular cloning of the DNA and expression and characterization of rat testes fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. *J. Biol. Chem.* **266**, 15764-15770
7. Ventura, F., Rosa, J.L., Ambrosio, S., Pilkis, S.J., and Bartrons, R. (1992) Bovine brain 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Evidence for a neural-specific isozyme. *J. Biol. Chem.* **267**, 17939-19743
8. Furuya, E., Yokoyama, M., and Uyeda, K. (1982) Regulation of fructose-6-phosphate,2-kinase by phosphorylation and dephosphorylation: Possible mechanism for coordinated control of glycolysis and glycogenolysis. *Proc. Natl. Acad. Sci. USA* **79**, 325-329
9. El-Maghrabi, M.R., Claus, T.H., Pilkis, J., and Pilkis, S.J. (1982) Regulation of 6-phosphofructo-2-kinase activity by cyclic AMP-dependent phosphorylation. *Proc. Natl. Acad. Sci. USA* **79**, 315-319
10. van Schaftingen, E., Davies, D.R., and Hers, H.G. (1981) Inactivation of phosphofructokinase 2 by cyclic AMP-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **103**, 362-368
11. Sakakibara, R., Kitajima, S., and Uyeda, K. (1984) Differences in kinetic properties of phospho and dephospho forms of fructose-6-phosphate,2-kinase and fructose 2,6-bisphosphatase. *J. Biol. Chem.* **259**, 41-49
12. Kitamura, K., Uyeda, K., Kangawa, K., and Matsuo, H. (1989) Purification and characterization of rat skeletal muscle fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. *J. Biol. Chem.* **264**, 9799-9806
13. Kitamura, K. and Uyeda, K. (1987) The mechanism of activation of heart fructose 6-phosphate,2-kinase:fructose-2,6-bisphosphatase. *J. Biol. Chem.* **262**, 679-681
14. Kitamura, K., Kangawa, K., Matsuo, H., and Uyeda, K. (1988) Phosphorylation of myocardial fructose-6-phosphate,2-kinase: fructose-2,6-bisphosphatase by cAMP-dependent protein kinase and protein kinase C. Activation by phosphorylation and amino acid sequences of the phosphorylation sites. *J. Biol. Chem.* **263**, 16796-16801

15. Sakai, A., Kato, M., Fukasawa, M., Ishiguro, M., Furuya, E., and Sakakibara, R. (1996) Cloning of cDNA encoding for a novel isozyme of fructose 6-phosphate,2-kinase/fructose 2,6-bisphosphatase from human placenta. *J. Biochem.* **119**, 506-511
16. Tabor, S. and Richardson, C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **82**, 1074-1078
17. Tominaga, N., Minami, Y., Sakakibara, R., and Uyeda, K. (1993) Significance of the amino terminus of rat testis fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. *J. Biol. Chem.* **268**, 15951-15957
18. Uyeda, K., Furuya, E., and Luby, L.J. (1981) The effect of natural and synthetic D-fructose 2,6-bisphosphate on the regulatory kinetic properties of liver and muscle phosphofructokinases. *J. Biol. Chem.* **256**, 8394-8399
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of head bacteriophage T4. *Nature* **227**, 680-685
20. Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
21. Pilkis, S.J., El-Maghrabi, M.R., and Claus, T.H. (1988) Hormonal regulation of hepatic gluconeogenesis and glycolysis. *Annu. Rev. Biochem.* **57**, 755-783
22. Kitamura, K. and Uyeda, K. (1988) Purification and characterization of myocardial fructose-6-phosphate,2-kinase and fructose-2,6-bisphosphatase. *J. Biol. Chem.* **263**, 9027-9033
23. Pilkis, S.J., Walderhaug, M., Murray, K., Beth, A., Venkataramu, S.D., Pilkis, J., and El-Maghrabi, M.R. (1983) 6-Phosphofructo-2-kinase/fructose 2,6-bisphosphatase from rat liver. *J. Biol. Chem.* **258**, 6135-6141
24. Pilkis, S.J., Regan, D.M., Stewart, H.B., Pilkis, J., Pate, T.M., and El-Maghrabi, M.R. (1984) Evidence for two catalytic sites on 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase. *J. Biol. Chem.* **259**, 949-958
25. Hasemann, C.A., Istavan, E.S., Uyeda, K., and Deisenhofer, J. (1996) The crystal structure of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase reveals distinct domain homologies. *Structure* **4**, 1017-1029
26. Lin, K., Li, L., Correia, J.J., and Pilkis, S.J. (1992) Arg-257 and Arg-307 of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase bind the C-2 phospho group of fructose-2,6-bisphosphate in the fructose-2,6-bisphosphatase domain. *J. Biol. Chem.* **267**, 19163-19171