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Biochemistry

Cytosolic thyroid hormone-binding protein is a monomer of pyruvate kinase

(fructose-1,6-bisphosphate/ADP/ATP/thermogenesis/enzyme regulation)

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ABSTRACT **A cDNA clone encoding a human cytosolic thyroid hormone-binding protein (p58) has been isolated. The human sequence was found to be homologous to that of rat pyruvate kinase (EC 2.7.1.40) subtype M_2. p58 is a monomer that has ≈5% the enzymatic activity of the tetrameric pyruvate kinase M_2. The tetrameric M_2 does not bind 3,3′,5-triiodo-L-thyronine (T_3). Binding of p58 to T_3 and its analogs resulted in the inhibition of its pyruvate kinase activity. The apparent K_i values of T_3, L-thyroxine, and D-T_3 are 30 nM, 100 nM, and 2 mM, respectively. L-Thyronine and 3,3′,5′-triiodo-L-thyronine had no effect. This order of activity correlates with the thermogenic effects reported for T_3 and its analogs. Conversion of p58 to the tetramer is reversible and is under the control of fructose 1,6-bisphosphate. The conversion is inhibited by T_3 in a dose-dependent manner. Since pyruvate kinase is a key enzyme in regulating cellular ADP, ATP, and pyruvate, our findings suggest that p58 may be involved in mediating some of the cellular metabolic effects induced by thyroid hormones.**

The thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) plays an essential role in maintaining fetal and neonatal development, regulating amino acid and electrolyte transport into the cell, modulating carbohydrate, protein, and lipid metabolism, and increasing the rate of oxidative phosphorylation. No single mechanism has been found to account for all the diverse actions of thyroid hormones.

Some of the thyroid hormone effects are initiated through the interaction of T_3 with nuclear receptors (see reviews in refs. 1 and 2). However, some of the effects of T_3, such as the enhancement of enzymatic activity (3, 4) and cellular amino acid accumulation and ATP synthesis (5) that occur in the absence of protein synthesis, have to be accounted for by alternative mechanism(s). Therefore, extranuclear T_3 binding sites that might mediate such activities have been sought. Cheng and coworkers (6) purified a cytosolic thyroid hormone-binding protein (p58) to homogeneity. The purified protein retains its T_3-binding activity and specificity (6), and two monoclonal antibodies against p58 have been developed (7). p58 has an apparent molecular weight of 60,000 by gel filtration and 58,000 by SDS/PAGE. To study its cellular function and the roles it plays in T_3 action, we isolated and sequenced the cDNA that encodes p58.‡ We found that p58 is a monomer of pyruvate kinase (ATP:pyruvate O^2-phosphotransferase, EC 2.7.1.40) subtype M_2 and that its conversion to the tetrameric pyruvate kinase is regulated by fructose 1,6-bisphosphate (Fru-1,6-P_2).

EXPERIMENTAL PROCEDURES

Materials. [^{125}I]T_3 (2200 Ci/mmol; 1 Ci = 37 GBq), L-[^{35}S]methionine (1097 Ci/mmol), and the reticulocyte lysate translation kit were from DuPont/NEN. Restriction enzymes and competent *Escherichia coli* HB101 cells were from BRL. The cDNA library constructed in λgt11 from human thyroid carcinoma was purchased from Clontech.

Preparation and Sequencing of a CNBr Fragment of p58. Purified p58 (6) (3 nmol of lyophilized powder) was dissolved in 0.5 ml of 70% formic acid containing 2% (wt/vol) CNBr and was incubated at 37°C for 1 hr. Electrophoresis of peptides and blotting onto poly(vinylidene difluoride) membrane for amino acid sequencing were carried out according to Matsudaira (8).

Isolation and Sequence Determination of cDNA Encoding p58. Screening of λgt11 phage plaques for p58 was carried out as described (9). The monoclonal antibodies used in the first and second screenings were J11 and J12, respectively (7). The phage containing the longest insert [2.1 kilobases (kb), TC6] was restricted with *Eco*RI and the insert was subcloned into pBluescript SK(+) (Stratagene). A *Pst* I fragment of this insert was labeled with ^{32}P and used to rescreen the library, and a longer insert (2.3 kb, TCB) was obtained. This insert was subcloned into pBluescript SK(+) as above.

Nucleotide sequence was determined by using single-stranded DNA prepared from TC6 and TCB as templates. Oligomers of 17–19 bases were synthesized from the preceding determined sequences and used as primers to determine the subsequent sequences. Both strands were sequenced.

***In Vitro* Transcription, Translation, and Immunoprecipitation.** TC6 was treated with *Sac* I and the resulting linearized plasmid was transcribed according to Cheng *et al.* (9). *In vitro* translation was carried out by using the rabbit reticulocyte lysate system according to the manufacturer's specifications. mRNA (500 ng) was used for *in vitro* translation. Aliquots (15 μl) of the mixture were analyzed by SDS/10% PAGE. For immunoprecipitation, the translation mixture was added to 0.3 ml of 0.14 M NaCl/0.03 M phosphate, pH 7.4, containing 0.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Monoclonal antibodies J11 and J12 were used for immunoprecipitation (7).

Determination of Enzymatic Activity and [^{125}I]T_3 Binding. Pyruvate kinase activity was measured by the 2,4-dinitrophenylhydrazone and NADH/lactate dehydrogenase coupled methods (10). Absorbance measurements were made in a Cary 15 spectrophotometer in quartz cuvettes thermostated at 25°C. All reagents were from Sigma. Binding was assayed by incubating purified protein (0.1 μg) or the cytosolic fraction (10 μg of protein) with 0.2 nM [^{125}I]T_3 for 30 min at 4°C. Protein-bound [^{125}I]T_3 was separated from unbound radioligand on a Sephadex G-25 (fine) column (6).

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Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; reverse-T_3, 3,3′,5′-triiodo-L-thyronine; T_4, L-thyroxine; Fru-1,6-P_2, fructose 1,6-bisphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M26252).

RESULTS

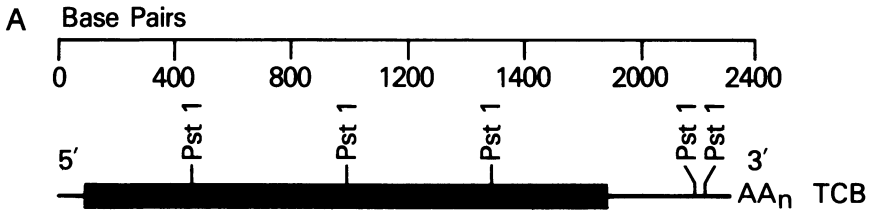
Partial Amino Acid Sequence of p58. To determine the partial amino acid sequence of p58, we attempted N-terminal sequence analysis of purified intact p58, but the N terminus was found to be blocked. We therefore cleaved the protein with CNBr. Six peptides of M_r 34,000, 32,000, 20,000, 16,000, 13,000, and 8600 were separated by SDS/5–18% gradient PAGE. The N-terminal sequence of the most abundant peptide (M_r 8600) was found to be NVAXLNFSHGTXEYX-AETIKNVXTATESF (X represents amino acids that could not be identified). Attempts were also made to determine the N-terminal sequence of the M_r 34,000 and 16,000 peptides, but the N termini of these two peptides were blocked. These results indicated that the M_r 34,000 and 16,000 peptides are partial cleavage products and contain the N terminus of p58.

Nucleotide Sequence Encoding p58. Two cDNA clones, TC6 and TCB, containing the coding region of p58 were isolated. TCB is 79 base pairs (bp) longer than TC6 and has the polyadenylylation site and sequence (Fig. 1*A*), which are absent in TC6. Fig. 1*B* shows the complete nucleotide sequence of TCB. The sequence is 2311 bp long and contains an open reading frame of 1593 bp with an initiation codon at nucleotide position 90 and a termination codon at 1683. The open reading frame encodes a protein of 531 amino acids with a calculated molecular weight of 57,978. This molecular weight is the same as that determined by SDS/PAGE (6). The open reading frame is preceded by a short (89-bp) 5′ untranslated region and a long (521-bp) 3′ untranslated region. A polyadenylylation signal is located at nucleotide 2285.

Examination of the deduced sequence indicated that TCB encodes p58. The partial amino acid sequence of the M_r 8600 peptide determined by Edman degradation was found in the deduced sequence at amino acids 70–98 (Fig. 1*B*). The calculated molecular weight of this CNBr-cleavable peptide (amino acids 70–149) is 8763, which agrees with that determined by SDS/PAGE. Examination of the deduced sequence further indicated that the M_r 34,000 and 16,000 peptides derived from partial CNBr cleavage most likely correspond to the deduced amino acid sequences 1–312 and 1–149. The calculated molecular weights of these two peptides are 34,203 and 16,302. Both peptides contain the N terminus of p58.

Size of the mRNA. Poly(A)$^+$ RNAs were isolated from two human epidermoid carcinoma cell lines (KB and A-431), electrophoretically fractionated, blotted, and hybridized with a 519-bp *Pst* I fragment (nucleotide positions 475–994) from TCB (Fig. 1*A*). One mRNA species of ≈2.4 kb was found (data not shown). The size of the mRNA confirmed that we had isolated a full-length cDNA for p58. The p58 mRNA was more abundant in KB cells than in A-431 cells. Earlier, p58 in the two human cell lines had been measured by [^{35}S]methionine metabolic labeling followed by immunoprecipitation with J11 and J12. Consistent with the mRNA levels , KB cells had a higher amount of labeled p58 than did A-431 cells (7).

Translation of *in Vitro* Transcribed mRNA for p58. Expression studies were carried out to determine whether TC6 encodes p58. The product of TC6 was first characterized by *in vitro* translation. TC6 was subcloned into the *Eco*RI site of pBluescript SK(+) phagemid that had been linearized by *Sst* I. Transcripts synthesized by phage T7 polymerase were translated *in vitro* using rabbit reticulocyte lysate. The [^{35}S]methionine-labeled products were analyzed by SDS/PAGE. TC6-4, a construct that has the insert in the opposite (antisense) orientation, was treated similarly. One major protein (M_r 58,000) and two very minor proteins (M_r 48,000 and 43,000) were detected for the TC6 (sense) plasmid, whereas the TC6-4 (antisense) plasmid did not yield any



B

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PKM2
p58                                                                                                  Pro      Asp
                                                                                                     MetSerLysProHisSerGluAlaGlyThr   10
     GGGCGGGAGCTGCACGCGCCGTGGCTCCGGATCTCTTCGTCTTTGCAGCGTACGCCCGAGTCGGTCAGCGCCGGAGGACCTCAGCAGCCATGTCGAAGCCCCATAGTGAAGCCGGGACT  119
PKM2                                                                                                 Ala
p58  AlaPheIleGlnThrGlnGlnLeuHisAlaAlaMetAlaAspThrPheLeuGluHisMetCysArgLeuAspIleAspSerProProIleThrAlaArgAsnThrGlyIleIleCysThr   50
     GCCTTCATTCAGACCCAGCAGCTGCACGCAGCCATGGCTGACACATTCCTGGAGCACATGTGCCGCCTGGACATTGATTCACCACCCATCACAGCCCGGAACACTGGCATCATCTGTACC  239
PKM2                 Met
p58  IleGlyProAlaSerArgSerValGluThrLeuLysGluMetIleLysSerGlyMetAsnValAlaArgLeuAsnPheSerHisGlyThrHisGluTyrHisAlaGluThrIleLysAsn   90
     ATTGGCCCAGCTTCCCGATCAGTGGAGACGTTGAAGGAGATGATTAAGTCTGGAATGAATGTGGCTCGCTGAACTTCTCTCATGGAACTCATGAGTAGCATGCGGAGACCATCAAGAAT  359
PKM2          Ala                 Ile
p58  ValArgThrAlaThrGluSerPheAlaSerAspProTyrLeuTyrArgProValAlaValAlaLeuAspThrLysGlyProGluIleArgThrGlyLeuIleLysGlySerGlyThrAla  130
     GTGCGCACAGCCACGGAAAGCTTTGCTTCTGACCCCTACCTCTACCGGCCCGTTGCTGTGGCTCTAGACACTAAAGGACCTGAGATCCGGAACTGGGCTCATCAAGGGCAGCGGCACTGCA  479
PKM2      Val
p58  GluLeuGluLeuLysLysGlyAlaThrLeuLysIleThrLeuAspAsnAlaTyrMetGluLysCysAspGluAsnIleLeuTrpLeuAspTyrLysAsnIleCysLysValValGluVal  170
     GAGCTGGAGCTGAAGAAGGGAGCCACTCTCAAAATCACGCTGGATAACGCCTACATGGAAAAGTGTGACGAGAACATCCTGTGGCTGGACTACAAGAACATCTGCAAGGTGGTGGAAGTG  599
PKM2                                                Glu      Tyr
p58  GlySerLysIleTyrValAspAspGlyLeuIleSerLeuGlnValLysGlnLysGlyAlaAspPheLeuValThrGluValGluAsnGlyGlySerLeuGlySerLysLysGlyValAsn  210
     GGCAGCAAGATCTACGTGGATGATGGGCTTATTTCTCTCCAGGTGAAGCAGAAAGGTGCCGACTTCCTGGTGACGGAGGTGGAAAATGGTGGCTCCTTGGGCAGCAAGAAGGGTGTGAAC  719
PKM2                                                                                  Leu                Ala
p58  LeuProGlyAlaAlaValAspLeuProAlaValSerGluLysAspIleGlnAspLeuLysPheGlyValGluGlnAspValAspMetValPheAlaSerPheIleArgLysAlaSerAsp  250
     CTTCCTGGGGCTGCTGTGGACTTGCCTGCTGTGTCGGAGAAGGACATCCAGGATCTGAAGTTTGGGGTCGAGCAGGATGTTGATATGGTGTTTGCGTCATTCATCCGCAAGGCCATCTGAT  839
PKM2
p58  ValHisGluValArgLysValLeuGlyGluLysGlyLysAsnIleLysIleIleSerLysIleGluAsnHisGluGlyValArgArgPheAspGluIleLeuGluAlaSerAspGlyIle  290
     GTCCATGAAGTTAGGAAGGTCCTGGGAGAGAAGGGAAAGAACATCAAGATTATCAGCAAAATCGAGAATCATGAGGGGGTTCGGAGGTTTGATGAAATCCTGGAGGCCAGTGATGGGATC  959
PKM2
p58  MetValAlaArgGlyAspLeuGlyIleGluIleProAlaGluLysValPheLeuAlaGlnLysMetMetIleGlyArgCysAsnArgAlaGlyLysProValIleCysAlaThrGlnMet  330
     ATGGTGGCTCGTGGTGATCTAGGCATTGAGATTCCTGCAGAGAAGGTCTTCCTTGCTCAGAAGATGATGATTGGACGGTGCAACCGAGCTGGGAAGCCTGTCATCTGTGCTACTCAGATG 1079
PKM2                                                                                  Thr
p58  LeuGluSerMetIleLysLysProArgProThrArgAlaGluGlySerAspValAlaAsnAlaValLeuAspGlyAlaAspCysIleMetLeuSerGlyGluThrAlaLysGlyAspTyr  370
     CTGGAGAGCATGATCAAGAAGCCCCGCCCCACTCGGGCTGAAGGCAGTGATGTGGCCAATGCAGTCCTGGATGGAGCCGACTGCATCATGCTGTCTGGAGAAACAGCCAAAGGGGACTAT 1199
PKM2
p58  ProLeuGluAlaValArgMetGlnHisLeuIleAlaArgGluAlaGluAlaAlaIleTyrHisLeuGlnLeuPheGluIleLeuArgArgLeuAlaProIleThrSerAspProThrGlu  410
     CCTCTGGAGGCTGTGCGGATGCAGCACCTGATTGCCCGTGAGGCAGAGGCTGCCATCTACCACTTGCAATTATTTGAGGAACTCCGGCGGCTGGCGCCCATTACCAGCGACCCCACAGAA 1319
PKM2 Ala
p58  AlaThrAlaValGlyAlaValGluAlaSerPheLysCysCysSerGlyAlaIleIleValLeuThrLysSerGlyArgSerAlaHisGlnValAlaArgTyrArgProArgAlaProIle  450
     GCCACCGCCGTGGGTGCCGTGGAGGCCTCCTTCAAGTGCTGCAGTGGGGCCATAATCGTCCTCACCAAGTCTGGCAGGTCTGCTCACCAGGTGGCCAGATACCGCCCACGTGCCCCCATC 1439
PKM2                                                                                  Ala      LeuAsp
p58  IleAlaValThrArgAsnProGlnThrAlaArgGlnAlaHisLeuTyrArgGlyIlePheProValLeuCysLysAspProValGlnGluAlaTrpAlaGluAspValAspLeuArgVal  490
     ATTGCTGTGACCCGGAATCCCCAGACAGCTCGTCAGGCCCACCTGTACCGTGGCATCTTCCCCTGTGCTGTGCAAGGACCCAGTCCAGGAGGCCTGGGCTGAGGACGTGGACCTCCGGGTG 1559
PKM2      Leu
p58  AsnPheAlaMetAsnValGlyLysAlaArgGlyPhePheLysArgGlyAspValValIleValLeuThrGlyTrpArgProGlySerGlyPheThrAsnThrMetArgValValProVal  530
     AACTTTGCCATGAATGTTGGCAAGGCCCGAGGCTTCTTCAAGAAGGGAGATGTGGTCATTGTGCTGACCGGATGGCGCCCTGGCTCCGGCTTCACCAACACCATGCGTGTTGTTCCTGTG 1679
PKM2
p58  ProEnd
     CCGTGATGGACCCCAGAGCCCCTCCTCCAGCCCCTGTCCCACCCCCTTCCCCCAGCCCATCCATTAGGCCAGCAACGCTTGTAGAACTCACTCTGGGCTGTAACGTGGCACTGGTAGGTT 1799

p58  GGGACACCAGGGAAGAAGATCAACGCCTCACTGAAACATGGCTGTGTTTGCAGCCTGCTCTAGTGGGACAGCCCAGAGCCTGGCTGCCCATCATGTGGCCCCACCCAATCAAGGGAAGAA 1919
     GGAGGAATGCTGGACTGGAGGCCCCTGGAGCCAGATGGCAAGAGGGTGACAGCTTCCTTTCCTGTGTGTACTCTGTCCAGTTCCTTTAGAAAAAATGGATGCCCAGAGGACTCCCAACCC 2039
     TGGCTTGGGGTCAAGAAACAGCCAGCAAGAGTTAGGGGCCTTAGGGCACTGGGCTGTTGTTCCATTGAAGCCGACTCTGGCCCTGGCCCTTACTTGCTTCTCTAGCTCTCTAGGCCTCTC 2159
     CAGTTTGCACCTGTCCCCACCCTCCACTCAGCTGTCCTGCAGCAAACACTCCACCCTCCACCTTCCATTTTCCCCCACTACTGCAGCACCTCCAGGCCTGTTGCTATAGAGCCTACCTGT 2279
     ATGTCAATAAACAACAGCTGAAGCACCAAAAA                                                                                          2311
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FIG. 1. Restriction map of the TCB cDNA insert (*A*) and nucleotide and predicted amino acid sequence of human cytosolic thyroid hormone-binding protein (*B*). In *B*, the amino acids that were determined by Edman degradation of the purified CNBr fragment are boxed. The polyadenylylation signal is underlined. The amino acids of rat pyruvate kinase subtype M_2 (PKM$_2$) that differ from the human p58 are listed for comparison. The DNA sequence was prepared using a computer program described by Shapiro and Senapathy (11).

protein (data not shown). Whether the M_r 58,000 protein synthesized *in vitro* was identical to that synthesized in A-431 cells was evaluated by immunoprecipitation and peptide mapping. The [^{35}S]methionine-labeled *in vitro* translation products were specifically immunoprecipitated by antibodies J11 and J12 (data not shown). The immunoprecipitable bands had the same apparent molecular weights as those obtained from [^{35}S]methionine-labeled A-431 cell lysate. The J11-immunoprecipitable band from *in vitro* translation and from A-431 cell lysate was excised and fragmented by *in situ* CNBr cleavage. Identical SDS/PAGE maps were obtained, indicating that the *in vitro* translated protein is the same as that synthesized in A-431 cells (data not shown). These results further confirmed that TC6 cDNA encodes p58.

Whether TC6 encodes a protein that binds T_3 was initially evaluated using the *in vitro* translation products of the TC6 transcript in reticulocyte lysate. However, because of the endogenous pyruvate kinase in the lysate, it was impossible to obtain unambiguous results. An expression vector was constructed that contains the coding region of p58 (nucleotides 90–2278), a T7 promoter, and a Shine–Dalgarno sequence. Upon induction by isopropyl β-D-thiogalactopyranoside, large amounts of p58 were expressed in *E. coli*. The expressed p58 bound T_3 specifically. The detailed characterization of the p58 expressed in *E. coli* will be described elsewhere. These results further confirmed that TC6 encodes p58.

p58 Is a Monomer of Pyruvate Kinase. A search against the GenBank data base (February 1988) revealed that p58 is homologous to the subunit of pyruvate kinase subtype M_2. There are four pyruvate kinase isoenzymes, L, R, M_1, and M_2, in mammalian systems. The L form is present mostly in liver, the R form exclusively in erythrocytes, and M_1 mostly in muscle; M_2 is found in many tissues and is increased in regenerating liver and liver of tumor-bearing animals (10). The L and R isozymes differ from the M_1 and M_2 forms in their electrophoretic, kinetic, and immunological properties. cDNAs and genomic clones for rat L, R, M_1, and M_2 have been isolated and sequenced (12, 13). Recently, the cDNA for the human L-type pyruvate kinase was reported (14). The L- and R-type and the M_1- and M_2-type isozymes are under the control of different genes. The L and R types of rat pyruvate kinase are produced from a single gene by use of different promoters, whereas the M_1 and M_2 types are produced from another single gene by alternative RNA splicing. Sequence analysis of human TCB and rat isozymes indicated that TCB belongs to the M_1/M_2 group. However, it has a higher sequence similarity to M_2: 91% at the nucleotide level and 97% at the amino acid level. The amino acids of rat M_2 pyruvate kinase that differ from those of human p58 are shown in Fig. 1*B*.

p58 Has Intrinsic Pyruvate Kinase Activity and Its Activity Is Inhibited by Thyroid Hormones. Pyruvate kinase is a very well-characterized enzyme. The isozymes consist of four identical subunits of M_r 57,000–62,000. The four isozymes have been isolated from many tissues of different species. In all cases, pyruvate kinases were isolated as a homotetramer. However, we have isolated and purified p58 with M_r 58,000. Since p58 is homologous to pyruvate kinase M_2, we evaluated whether p58 possesses pyruvate kinase activity. At 25°C, purified p58 had pyruvate kinase activity of 6 ± 0.6 units/mg (mean ± SD, n = 5). We determined the activity of two commercial pyruvate kinases for comparison: the rabbit and chicken enzymes had specific activities of 108–125 units/mg, similar to the reported values (15).

The effect of various concentrations of T_3 and its analogs on the pyruvate kinase activity of purified p58 was evaluated (Fig. 2). T_3, T_4, and D-T_3 inhibited the pyruvate kinase activity with apparent K_i values of 30 nM, 100 nM, and 2 mM, respectively. In contrast, L-thyronine and reverse-T_3 had no effect on the activity of the monomer. These results indicate that the inhibition is thyroid hormone-specific.

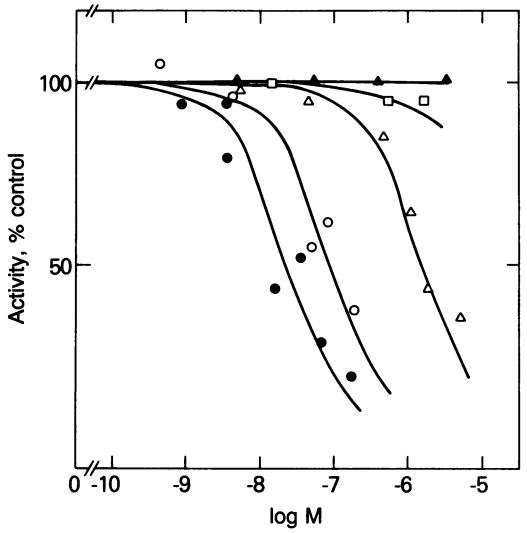

FIG. 2. Dose-dependent inhibition of pyruvate kinase activity by thyroid hormones and their analogs. Purified p58 (12.5 μg/ml) was preincubated with various concentrations of T_3 (●), L-thyroxine (T_4, ○), D-T_3 (△), D-T_4 (□), or 3,3',5'-triiodo-L-thyronine (reverse-T_3, ▲) for 2 hr at 4°C. An aliquot (0.25 μg/20 μl) of the p58 solution was added to the 1-ml assay and the activity was determined.

Regulation of the Formation of the Cellular p58 by Fru-1,6-P_2. It was intriguing that we had isolated a monomer of pyruvate kinase from A-431 cells rather than a tetramer, as isolation of the monomer from tissues or cells had never been reported. A comparison of the buffers used in the purification of p58 and the buffers commonly used in the preparation of pyruvate kinase revealed that the major difference was the absence of Fru-1,6-P_2 in our buffers. Pyruvate kinase is activated by Fru-1,6-P_2 (10), though the mechanism of activation is unknown. We postulated that p58 is in equilibrium with the tetrameric enzyme *in vivo* and that in the absence of Fru-1,6-P_2, the formation of the tetramer is inhibited and the p58 monomer is stabilized. To test this, we compared the pyruvate kinase and T_3 binding activities in the cytosolic fraction of cells prepared in the presence or absence of Fru-1,6-P_2. In the presence of Fru-1,6-P_2, the peak of pyruvate kinase activity was located at fraction 28 of a Sephadex G-200 column where the standard tetrameric pyruvate kinase was also located (Fig. 3*A*). The specific activity was 42 ± 6 units/mg of protein (mean ± SD, n = 4). Examination of all fractions failed to detect specific T_3 binding activity.

In the absence of Fru-1,6-P_2, low pyruvate kinase activity (2.9 ± 0.5 units/mg, mean ± SD, n = 4) was detected in fraction 36 and no activity was detected in fraction 28 (Fig. 3*B*). However, T_3 binding activity was also detected in fraction 36. When the G-200 column was calibrated with purified p58, the peak of T_3 binding activity was found in fraction 36. Therefore, the pyruvate kinase activity detected in fraction 36 was due to the intrinsic enzymatic activity of P58. To be sure that the tetramer detected in fraction 28 in the presence of Fru-1,6-P_2 and p58 detected in fraction 36 in the absence of Fru-1,6-P_2 were from the same protein, an immunoblot experiment was carried out using antibody J11. The peak of J11 reactivity was at fraction 28 in the presence of Fru-1,6-P_2 (Fig. 3*A*) and at fraction 36 in its absence (Fig. 3*B*). No J11 reactivity was detected in fractions 36 and 28 in the presence and absence of Fru-1,6-P_2, respectively. Thus, the tetramer does not bind T_3 whereas the monomer does. Further, in the absence of Fru-1,6-P_2, p58 is stabilized and does not associate to form tetrameric pyruvate kinase.

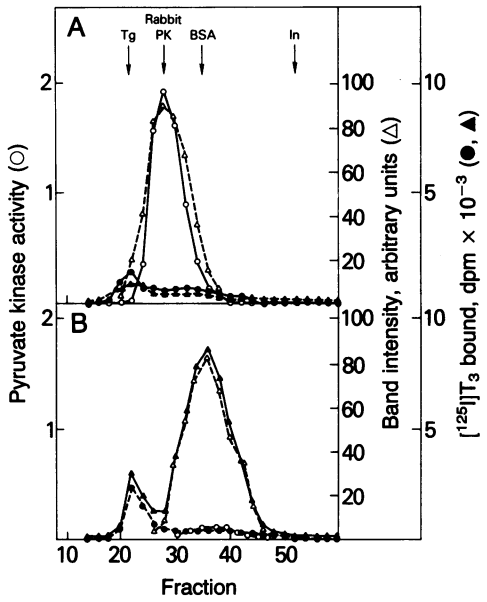

FIG. 3. Sephadex G-200 column chromatography of the cytosolic fraction of A-431 cells. A-431 cells were plated at 5×10^6 per 150-mm dish. After incubation for 20 hr, cells were washed, pelleted, and sonicated in phosphate-buffered saline in the presence (*A*) or absence (*B*) of 5 mM Fru-1,6-P_2. An aliquot (2 mg of protein) of lysate was applied to a G-200 column (0.8 × 48 cm) and fractionated with a flow rate of 2 ml/hr. The fractions were analyzed for pyruvate kinase activity (○), for [^{125}I]T_3 binding activity in the presence (●) or absence (▲) of 1 μM unlabeled T_3, and for immunoreactivity with J11 by Western blotting (△). The column was calibrated with thyroglobulin (Tg), rabbit pyruvate kinase (PK), bovine serum albumin (BSA), and insulin (In).

The above results provided us with a convenient way to assay the formation of p58 and the tetrameric pyruvate kinase. We determined the effective concentration of Fru-1,6-P_2 that facilitates the association of p58 to form the tetramer. Cytosolic fractions of A-431 cells were prepared in the presence of various concentrations of Fru-1,6-P_2, and T_3 binding and enzymatic activity were determined. Fig. 4 shows the reciprocal relationship between T_3 binding and the tetrameric enzyme activity. At 100 μM Fru-1,6-P_2, 40% loss of T_3 binding activity was accompanied by the regeneration of ≈30% of the tetrameric enzyme activity. At 5 mM Fru-1,6-P_2, complete loss of T_3 binding activity was concurrent with complete recovery of enzyme activity. In a separate experiment, we prepared the cytosolic fraction in the presence of 5 mM Fru-1,6-P_2. As expected, no T_3 activity was detected. After removal of Fru-1,6-P_2 by dialysis, T_3 binding activity was regained and the tetrameric pyruvate kinase activity was lost. When the cytosolic fraction was prepared in the absence of Fru-1,6-P_2, T_3 binding activity was detected, but pyruvate kinase activity was not. After the addition of 5 mM Fru-1,6-P_2, enzymatic activity was found and T_3 binding activity was lost. These results indicate that p58 and the tetrameric pyruvate kinase are in a dynamic equilibrium that is functionally regulated by Fru-1,6-P_2.

In earlier work it was found that CHAPS stabilized the T_3 binding activity of p58; therefore CHAPS was used in the buffers during purification. To see whether CHAPS also dissociates the tetramer to p58, we prepared the cytosolic fraction of A-431 cells in the presence of CHAPS and Fru-1,6-P_2. Up to 3 mM CHAPS, no loss of tetrameric enzyme activity was found. We concluded that CHAPS only acts to stabilize p58 once it is formed.

Fru-1,6-P_2-Induced Conversion of p58 to Pyruvate Kinase Is Inhibited by T_3. To understand the physiological consequence of T_3 binding to p58, we evaluated the effect of T_3 on

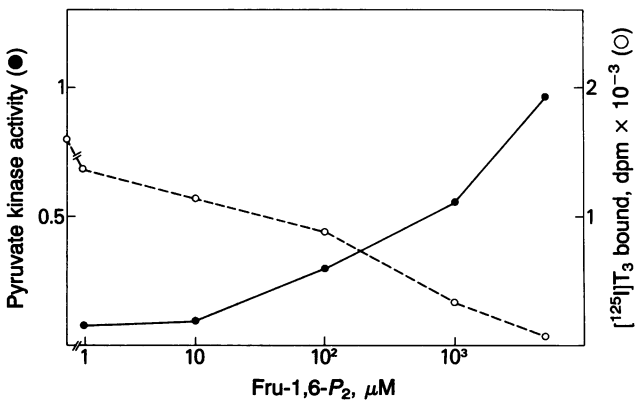

FIG. 4. Regulation of the p58–pyruvate kinase interconversion by Fru-1,6-P_2. The cytosolic fraction of A-431 cells were prepared as in Fig. 3 in the presence of various concentrations of Fru-1,6-P_2. Aliquots (1 μg of protein) of lysate were assayed for pyruvate kinase (●) and [^{125}I]T_3 binding (○) activity.

the conversion of p58 to pyruvate kinase in the physiological concentration range of Fru-1,6-P_2 (Fig. 5). T_3 inhibited the Fru-1,6-P_2-induced conversion of p58 to pyruvate kinase in a dose-dependent manner in the A-431 cell lysate. At 100 μM Fru-1,6-P_2, inhibition by 0.1, 1, and 1000 nM T_3 was 35%, 48%, and 100%, respectively. However, the inhibition could be overcome by increasing the concentration of Fru-1,6-P_2. At 5 mM Fru-1,6-P_2, the inhibition by 0.1, 1, and 1000 nM T_3 was reduced to 5%, 25%, and 49%, respectively. This inhibition was T_3-specific; reverse-T_3 at 0.1–1 nM had no effect and at 1 μM had only 5–10% the activity of T_3 (Fig. 5).

The inhibitory effect of T_3 on the conversion of p58 to pyruvate kinase was not only evident in the cytosolic fraction of A-431 cells but was also seen with purified p58. At 0.5 mM Fru-1,6-P_2, 50% of the conversion of p58 to the enzyme was inhibited by 1 nM T_3 (data not shown).

DISCUSSION

This paper reports the molecular cloning and sequence of a human cytosolic thyroid hormone-binding protein. From the analysis of protein sequence, size of mRNA, molecular size of protein, immunoreactivity, and peptide mapping, it is clear

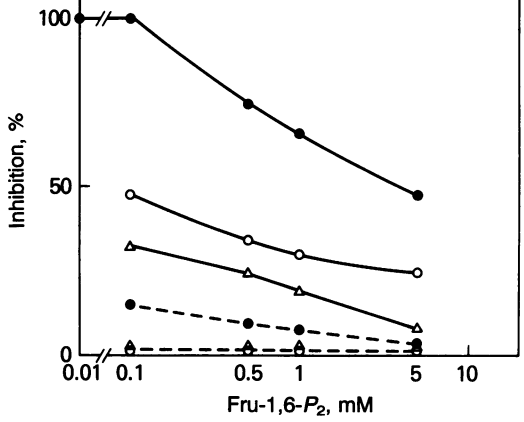

FIG. 5. Inhibition by T_3 of the Fru-1,6-P_2-induced conversion of p58 to pyruvate kinase. A-431 cell lysate was prepared in phosphate-buffered saline as described in Fig. 3. Aliquots (1 μg of protein) of lysate were incubated for 30 min at 4°C in the absence or presence of 1 μM (–●–), 1 nM (–○–), or 0.1 nM (–△–) T_3 or 1 μM (––●––), 1 nM (––○––), or 0.1 nM (––△––) reverse-T_3. After incubation of the lysate with 0.01–5 mM Fru-1,6-P_2, pyruvate kinase activity was evaluated by the 2,4-dinitrophenylhydrazone method (10).

that TCB encodes the p58 identified and purified earlier (6, 7). *In vitro* translation using rabbit reticulocyte lysate and mRNA prepared from cDNA yielded one major protein, with a size identical to that calculated from the deduced sequence indicating initiation from the ATG at nucleotide 90. Identical peptide maps from the *in vitro* translation product and the p58 purified from A-431 cells further indicated that this ATG is indeed the initiator. This conclusion was further supported by the p58 expressed in *E. coli*. The expression vector contains the coding sequence of p58 beginning at nucleotide 90 and the expressed p58 binds T_3.

Sequence comparison indicated that p58 is a monomer of pyruvate kinase M_2. The purified p58 not only binds T_3 but also has enzymatic activity (6 ± 0.6 units/mg of protein) that is $\approx 5\%$ that of the tetrameric pyruvate kinase. These results are in contrast to earlier reports in which the monomer was shown to lack enzymatic activity (16–18). In those studies, the monomer was obtained by using a denaturant, guanidinium chloride, which may have irreversibly inactivated the enzymatic activity of the monomer. In the present study, however, p58 was isolated in the presence of a zwitterionic detergent, CHAPS. CHAPS has been used in many studies as a mild detergent to stabilize proteins. Therefore, the enzymatic activity of the p58 was retained. We also have considered the possibility that the enzymatic activity detected for p58 might be due to a trace amount of reassociated tetramer. However, we ruled out this possibility in two ways. (*i*) Association of monomers to form tetrameric pyruvate kinase is time- and concentration-dependent (19). The enzymatic activity of p58 not only did not increase with time but decreased gradually at 22°C, whereas under the same conditions, the activity of the tetramer was unchanged. (*ii*) That the pyruvate kinase activity of p58 was inhibited by T_3 and its analogs in a dose-dependent manner further argues against this possibility. The tetrameric enzyme isolated from A-431 cells or obtained commercially does not bind T_3. Thus, p58 has intrinsic pyruvate kinase activity. The discovery that p58 has two functions is not without precedents. Protein disulfide isomerase has been found to be the same protein as the β subunit of proline-4-hydroxylase (20), the glycosylation binding site (21), the 5′-deiodinase (22), and a T_3 binding protein (9). Recently, the myosin light-chain kinase was found to bind T_4 selectively (23). The mannose-6-phosphate receptor is identical to the receptor for insulin-like growth factor I (24). More multifunctional proteins are certain to be discovered as more genes are cloned and their sequences determined.

Fru-1,6-P_2 is an activator of pyruvate kinase, but the mechanism of activation has never been elucidated. Our findings indicate that Fru-1,6-P_2 regulates the interconversion of monomer and tetramer of pyruvate kinase. When mammalian cells are cultured in medium containing a low concentration of glucose (50 μM), the cellular level of Fru-1,6-P_2 is low (25 μM). Under these conditions, pyruvate kinase is functionally inactive. No phospho*enol*pyruvate is converted to pyruvate, no ATP is synthesized in the glycolytic pathway, and no pyruvate is available for ATP production by pyruvate oxidation (25). On the other hand, in regular medium containing 5–10 mM glucose, high levels of Fru-1,6-P_2 (267–2800 μM) were found in proliferating and tumor cells, which require high pyruvate kinase activity for growth (25). These observations are entirely consistent with our present data that low concentration of Fru-1,6-P_2 favors the formation of p58 and high concentration of Fru-1,6-P_2 converts p58 to the tetrameric enzyme. Since the concentrations of Fru-1,6-P_2 we used were within the physiological range, this mode of regulation could conceivably be the regulatory mechanism of pyruvate kinase activity *in vivo*.

The thyroid hormones are known to increase oxygen consumption and heat production. In thyroidectomized rats, administration of T_3 increases ATP production in mitochondria and the increase is independent of protein synthesis (5). The present study showed that p58 is in equilibrium with pyruvate kinase. At low cellular concentration of Fru-1,6-P_2, p58 is formed, which binds T_3. As a result of T_3 binding, not only is the intrinsic enzymatic activity of p58 inhibited, but also its association to form tetrameric pyruvate kinase is blocked. The extent of this inhibition depends on the cytosolic concentrations of Fru-1,6-P_2 and T_3. The physiological cytosolic T_3 concentration is 1–10 nM (26). Our data indicate that 0.1–1 nM T_3 inhibits the formation of pyruvate kinase (Fig. 5). Therefore, *in vivo*, this inhibition could lead to a reduction of cellular pyruvate kinase activity. In intact Ehrlich ascites tumor cells, when pyruvate kinase is inhibited, intracellular phospho*enol*pyruvate and ADP accumulate while pyruvate and ATP decrease. Concomitant with these changes is a decrease in glycolytic rate and an increase in O_2 consumption (27). Thus, T_3 could act to increase O_2 consumption by a similar mechanism. The ADP accumulated as the result of lower pyruvate kinase activity would stimulate the rate of O_2 consumption. However, the nature of the involvement of p58 in thyroid hormone-induced metabolic effects will require further investigation.

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