Isozymes of Human Phosphofructokinase in Blood Cells and Cultured Cell Lines: Molecular and Genetic Evidence for a Trigenic System

By Shobhana Vora

We have previously demonstrated that human red cell (RBC) phosphofructokinase (PFK) is under dual genetic control, since it is a mixture of five tetrameric isozymes composed of two distinct subunits, M (muscle-type) and L (liver-type), i.e., M_4, M_3L, M_2L_2, M_L_3, and L_4. The residual RBC PFK in a patient with Tarui’s disease (glycogenosis type VII) was shown to consist exclusively of L_4-type isozyme, indicating homozygosity for the deficiency of M-type subunits. In this study, we have investigated various normal and M-deficient blood cells and cultured cell lines for their quantitative and qualitative PFK profiles, using monoclonal antibodies in addition to protein hybridization techniques. Our results indicate that erythrocytes and platelets exhibit the least PFK activities as compared to various nucleated blood cells. However, the highest activities are exhibited by cultured fibroblasts and lymphoblastoid cell lines. The lymphoblastoid cell line from our M-deficient patient exhibits a relative PFK deficiency as compared to the normal cell lines. The platelet PFK consists of three species with subunit structures of \( P_R \), \( P_L \), and \( P_{L2} \), since dissociation and reassociation of platelet

PFK yields a five-membered set. The “P” subunit is under separate genetic control, since it exhibits a unique chromatographic mobility, complete resistance to anti-M and anti-L monoclonal antibodies, and normal expression in Tarui’s disease. Lymphocytes and polymorphonuclear leukocytes exhibit predominantly L_4 isozyme with some hybrids consisting of all 3 subunits. Fibroblast PFK is resolved chromatographically into 10–12 species, five being identical to those of RBC and 3 to those of platelets, suggesting simultaneous expression of all 3 genes. The hybridization of RBC PFK (M + L) and platelet PFK (P + L) yields the same 10–12 fibroblastic species, proving expression of all 3 genes. Normal lymphoblastoid cell lines exhibit the same 10–12 species seen in fibroblasts, as do the RBC of a few otherwise healthy individuals. In accord with the above data, the lymphoblastoid cell line from the M-deficient patient exhibits only 5 species composed of P and L subunits. These studies demonstrate the multicomponent forms of PFK in various blood cells and cultured cell lines and provide evidence for the existence of 3 structural loci coding for human PFK, i.e., M, P, and L.

PHOSPHOFRUCTOKINASE (PFK; ATP: D-fructose-6-P. 1-phosphotransferase, EC. 2.7.1.11.) occupies a key position in the cellular control of glycolysis in most organisms. The first indication of the possible existence of isozymes of PFK came from the description of a recessively inherited deficiency state in humans characterized by exertional myopathy and compensated hemolysis, also called Tarui’s disease (glycogen storage disease type VII). In these patients, PFK activity was essentially absent from muscle, half-normal in red cells (RBC), and normal in white cells (WBC). The partial reduction of the RBC enzyme was explained by the postulate that normal RBC PFK is a heterogeneous protein and that 50% of its activity is due to “muscle-type” PFK protein. The proposed structural heterogeneity of RBC PFK was originally supported by immunologic studies and more recently by the direct demonstration of two nonidentical subunits, M (muscle type) and R or E (nonmuscle type). Using the techniques of protein hybridization and column chromatography, we recently have demonstrated that the nonmuscle subunit of RBC PFK is liver type (L) in nature and that the RBC enzyme is not a single hybrid isozyme, but a heterogeneous mixture of the five tetrameric isozymes, which result from the random polymerization of M- and L-type subunits: M_4, M_3L, M_2L_2, M_L_3, and L_4. In accord with these data, we demonstrated that the residual RBC PFK from a patient with Tarui’s disease consists exclusively of L_4-type isozyme, indicating a homozygosity for the deficiency of M subunits. However, the WBCs and platelets from our patient showed normal activities, indicating that the PFK isozymes from these cells are under genetic control(s) separate from that of the M subunit. These findings prompted us to undertake studies to elucidate the number and subunit structures of PFK isozymes present in various normal and M-deficient blood cells and cultured cell lines, as well as their relative PFK activity levels per cell basis. Our results indicate that three structural loci code for human PFK, and these are variably expressed in different blood cells and cultured cell lines. A preliminary report of these studies has been presented.

MATERIALS AND METHODS

Chemicals and Reagents

Adenine nucleotides, NADH, Fru-6-P, Fru-1,6-diphosphogluconate, sodium d-glycerol-P were purchased from Sigma Chemical Co., St. Louis, Mo. Aldolase, \( \alpha \)-glycerol-P-dehydrogenase,

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ase, triose-P-isomerase and other enzymes were from Boehringer Mannheim (Indianapolis, Ind.). Enzyme-grade ammonium sulfate was obtained from Schwarz-Mann (Spring Valley, N.Y.). DEAE-Cellulose (DE-52) was obtained from Whatman (Clifton, N.J.) and DEAE-Sephadex A-25 from Pharmacia (Piscataway, N.J.). RPMI 1640, Eagle’s MEM, and fetal calf serum (FCS) were products of GIBCO, Grand Island, N.Y. Isolymph, a Ficoll-Hypaque density gradient medium, was obtained from Gallard-Schlesinger Chemical Corp., Carle Place, N.Y. Polyethylene glycol was obtained from Baker (Phillipsburg, N.J.) and staphylococci bearing protein A (lgGSorb) from the Enzyme Center, Inc., Boston, Mass. All other chemicals were of reagent grade.

**Blood Products**

For RBC PFK extraction, recently outdated units of packed RBCs were obtained from the blood bank. For platelet PFK extraction, 50 units of recently outdated platelets stored at 4°C were obtained through the courtesy of the Greater New York Blood Center. For the determination of the enzyme activity levels and chromatographic and immunologic studies of PFKs from various cell types, fresh blood samples (heparinized and defibrinated) were obtained from patients and healthy volunteers of different ethnic backgrounds. These were stored at 4°C immediately after collection and processed within 4 hr.

**Preparation of Pure Cell Suspensions**

Pure RBC suspensions were obtained by filtration of saline-diluted defibrinated blood through two thicknesses of Whatman no. 2 filter paper twice. Pure granulocyte preparations were prepared according to the method described by Goldstein et al. except for one modification: the final preparation was subjected to a density gradient centrifugation step using 20.6% Stractan according to Corash et al. to remove contaminating platelets. Pure suspensions of total peripheral lymphocytes were obtained by centrifugation of saline-diluted blood (v:v) on Isolymph gradients according to the manufacturer’s instructions. Pure platelet suspensions were prepared by the method described by Corash et al. The final RBC, WBC, and platelet suspensions were counted by standard methods.

**Cell Lines and Cell Cultures**

Normal human cultured fibroblasts (early passage) derived from adult skin explants and fetal amniotic fluid were obtained from Drs. A. Miranda and D. Warburton, respectively (Departments of Pathology and Human Genetics, respectively, Columbia University College of Physicians and Surgeons, N.Y.). Amnion cell lines (GM 472, GM 957, and GM 956) and Epstein-Barr virus transformed lymphoblastoid cell lines (GM 605, GM 558, and GM 3201) were obtained from the Human Mutant Genetic Cell Repository, Camden, N.J. A long-term lymphoblastoid cell line (GM 3128) was established from our PFK-deficient patient through the courtesy of the Human Genetic Mutant Cell Repository, Camden, N.J. The fibroblasts and amnion cells were grown in large tissue culture flasks (Falcon, 150 sq cm) containing 100 ml of Eagle’s MEM with Earle’s Salts supplemented with 1% each of t-glutamine, penicillin, streptomycin, and nonessential amino acids, and 15% FCS at 37°C in 5% CO₂ and 95% air. Fibroblasts were transferred when cultures became confluent by dilution with fresh culture medium. All the lymphoblastoid cell lines were grown in plastic flasks containing RPMI 1640, supplemented with 1% each of t-glutamine, penicillin, streptomycin, and 15% FCS. The flasks were incubated at 37°C in 5% CO₂ and 95% air. The viable cells were counted by the dye-exclusion technique utilizing the standard manual method.

**Preparation of Cell Extracts**

Hemolysates were prepared just prior to chromatography by the addition of an equal volume of 10 mM potassium phosphate buffer (pH 8.0) containing 0.2 mM EDTA, 0.2 mM AMP, 0.7 mM diithiothreitol, and 0.2% Saponin to the packed RBCs. The WBCs, platelets, and cultured cells were washed 3 times in buffered isotonic saline and resuspended in small volumes (0.3-0.5 ml) of 50 mM potassium phosphate buffer (pH 8.0) containing 0.2 mM EDTA, 0.2 mM AMP, and 0.7 mM diithiothreitol. The cells were disrupted by sonication for 10 sec x 3, using a Branson sonifier (Model 185) at 30 W. The hemolysates and cell sonicates were centrifuged at 10,000 g for 10 min, and supernatants were used for assays, chromatography and immunoprecipitation experiments.

**Partial Purification of PFKs**

RBC PFK was purified according to the method described by Wenzel et al. except that the last two gel-filtration steps were substituted by a single DEAE-cellulose chromatography step according to Layzer et al. Platelet PFK was purified as follows. Platelets were washed and lysed according to Akkerman et al. The lysed preparation was centrifuged at 24,000 g for 20 min at 4°C to obtain the supernatant containing PFK, which was further subjected to the extraction procedure of Wenzel et al. except that the heat-treatment was modified (the preparation was allowed to reach only 56°C and then was immediately cooled to 0°C). The final preparation was subjected to DE-52 chromatography after dialysis, according to Layzer et al.

**Production of Anti-M and Anti-L Monoclonal Antibodies**

Subunit-specific monoclonal antibodies against M and L subunits of RBC PFK were produced by the secretory lymphocyte hybridoma technique of Kohler and Milstein using immunized spleen cells from BALB/c mice and a thioquinine-resistant BALB/c myeloma cell line (45.6.TG.1.7.5), which synthesizes only α-chains and is killed by hypoxanthine-aminopterin and thymidine medium. The fusion was done using 3% polyethylene-glycol (PEG 1000, Baker) using a procedure similar to that described by Davidson and Geral. Cloning of antibody-secreting hybridomas was done in soft agarose according to the method described by Sharon, Morrison, and Kabat. Antibody-rich ascites fluids were raised by injecting hybridomas into the peritoneal cavities of CDFI (BALB/c x DBA/2) F₁ mice. The details of the immunization protocol, techniques of hybridization, screening of the secretony hybrids, and characterization of the secreted antibodies are described elsewhere.

**PFK Assays**

PFK assays were performed using a Gilford Model 250 spectrophotometer at 26°C as described earlier. One unit of enzyme is defined as that amount of enzyme that converts 1 μmole of F-6-P to Fru-1,6-P in 1 min in the above system per 10⁶ cells. At least three separate donors or cell lines were investigated in duplicate to establish the PFK content of each cell type.

**Chromatographic Separation of PFK Isozymes**

Chromatographic separation of PFK isozymes was obtained on a DEAE-Sephadex A-25 column equilibrated with 0.1 M Tris-P buffer (pH 8.0) containing 0.2 mM EDTA, 0.2 mM AMP, and 1 mM diithiothreitol. A concave gradient of NaCl was used for elution as previously described, except that to further amplify the resolution, 1.5-ml (instead of 3-ml) fractions were collected in the initial
one-half of the gradient. At least three separate donors or cell lines were investigated to establish the PFK profile of each cell-type.

**Dissociation–Reassociation Experiments**

These were performed using the method of Tsai and Kemp as previously modified. Partially purified platelet and RBC PFKs were dissociated separately by lowering the pH of the solutions from 8.0 to 5.2. For hybridization, desired amounts of these solutions were then mixed and reassociation was effected by adjusting the pH of the mixture to 7.2. Partially purified platelet PFK was also subjected to similar dissociating and reassociating conditions by itself. The dissociation and reassociation of isozymes were evidenced by loss and recovery of enzymatic activity, respectively.

**Immunologic Studies**

Enzyme precipitation studies using both types of monoclonal antibodies were performed by the modification of the immunoprecipitation assay technique described previously. Since the monoclonal antibodies were found to be nonprecipitating in nature, staphylococci bearing protein A (IgGSorb) were used to precipitate soluble antigen-antibody complexes. Cell extracts containing PFK were digested with 50 mM potassium fluoride (KF) buffer (pH 7.5), containing 5 mM EDTA, 3 mM DTT, 0.5 mM AMP, 0.5 mM Fru-6-P, and 5 mM (NH₄)₂SO₄, to a final concentration of 0.05–0.1 U/ml. In a series of test tubes, 0.05 ml of diluted PFK preparation was mixed with 0.05 ml of diluted mouse ascites fluid (1:200) containing either anti-L or anti-M antibody. The mixtures of enzymes and diluted ascites fluids were incubated at 37°C for 30 min and then 0.15 ml of reconstituted suspension of staphylococci (IgGSorb) was added. The tubes were incubated at 4°C for 1 hr with continuous shaking, and then centrifuged at 4000 g for 10 min at 4°C. The supernatants were assayed for residual enzyme activities. Concurrent controls were run using both nonimmune mouse serum as well as KF buffer. Each cell extract was run in duplicate on two different occasions against each antibody with concurrent controls as described above.

**RESULTS**

**Preparation of Pure Cell Suspensions**

A relatively high degree of purity was obtained for each of the four native cell types, i.e., RBC, polymorphonuclear leukocytes, lymphocytes, and platelets.

The purity of the cell suspensions was checked by performing differential cell counts as well as by morphological examinations on the final preparations. Pure RBC suspensions contained less than 0.003% and 0.01% contamination by WBC and platelets, respectively. Pure polymorphonuclear leukocyte and lymphocyte suspensions exhibited less than 5% contamination by lymphocytes and platelets, respectively; other cell types were absent, including monocytes. Pure platelet suspensions contained 0%–0.1% lymphocytes and RBC.

**PFK Activity Values**

Table 1 summarizes the PFK activity levels of various native and cultured cell types when expressed as units per 10⁹ cells. The order of increasing PFK activity per 10⁹ cells was as follows: platelets, RBC, neutrophils, lymphocytes, lymphoblastoid cell lines, cultured fibroblasts, and amnion cells. The normal lymphoblastoid cell lines exhibited an average PFK activity of 91 ± 7 U/10⁹ cells. In contrast, that from the patient exhibited an average of 49 ± 13 U/10⁹ cells.

**Chromatographic Separation of PFK Isozymes**

1) *From normal cells.* As shown in Fig. 1, RBC PFK is resolved into a five-membered isozyme set as described previously. The first and the last peaks elute in the positions of muscle and liver isozymes, respectively, and three hybrids in the intermediate positions. Platelet PFK elutes as three peaks in the general positions of RBC hybrids (M₁L, M₁L₂, and M₁L). P₄ elutes in the position very close to that of M₁L species. Lymphocytes and granulocytes exhibit predominantly the L₄ species (50%–60%); however, multiple additional minor peaks elute in the positions of the hybrid isozymes.

<table>
<thead>
<tr>
<th>Tissue or Cell Type</th>
<th>PFK Activity (U/10⁹ Cells)</th>
<th>Percent Precipitation (Mean ± 1SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-M</td>
</tr>
<tr>
<td>Muscle (6)</td>
<td>—</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>Liver (6)</td>
<td>—</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Platelets (9)</td>
<td>0.60 ± 0.19</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>Red Cells (6)</td>
<td>1.90 ± 0.07</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>Lymphocytes (6)</td>
<td>10.5 ± 0.40</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>Polymorphs (7)</td>
<td>12.0 ± 2.50</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>Lymphoblastoid cell line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (4)</td>
<td>91 ± 7</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>M-deficient (4)*</td>
<td>49 ± 13</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Amnion cells (3)</td>
<td>309 ± 60</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults (5)</td>
<td>289 ± 138</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>Fetal (4)</td>
<td>413 ± 133</td>
<td>51 ± 12</td>
</tr>
</tbody>
</table>

*Numbers in parentheses of separate donors or cell lines investigated.

*Number of observations on a single cell line.
Fig. 1. Chromatographic separation of PFK isozymes from various human blood cells, showing the presence of 5 isozymes in erythrocytes, 3 in platelets, and multiple in lymphocytes and neutrophils.

As shown in Fig. 2, the PFKs from the cultured fibroblasts and lymphoblastoid cell lines are consistently resolved into more than 5 (usually 10-12) isozymic species. Five of these are chromatographically identical to those of RBC isozymes and 3 are identical to those of platelets. The isozymic profiles from the RBCs of 13 individuals revealed two basic patterns; 9 individuals exhibited a five-membered set (Fig. 1), whereas 4 (2 of whom were siblings) exhibited 10-12 isozymes (Fig. 2).

(2) From M-deficient cells. Platelets and WBCs from our patient exhibit nearly normal isozymic profiles; however, as shown in Fig. 2, his lymphoblastoid cell line exhibits only 5 species, instead of the 10-12 exhibited by normal cell lines (Fig. 2). Three of these are indistinguishable from those present in platelets; the last one elutes in the position of L4, and one species just prior to L4. As reported earlier, his RBC exhibits only L4, with a total absence of the other 4 species.10

Dissociation- Reassociation Experiments

Dissociation and reassociation of partially purified platelet PFK produces a unique five-membered isozyme set (Fig. 3A), whereas the hybridization of platelet and RBC PFKs produces 10-12 chromatographic species (Fig. 3B).

Immunoprecipitation Studies

Table 1 summarizes the results of immunoprecipitation studies using two types of monoclonal antibodies. The monoclonal anti-M (V96-26) and anti-L (V65-06) antibodies exhibit exquisite specificity for the respective subunit and no cross-reactivity with the other two subunits.

(1) Anti-M antibody. An excess of this antibody completely precipitates muscle PFK, whereas the L4 homotetramer isolated either from liver or RBC is completely resistant. The PFKs from all the normal blood cells except platelets and cultured cell lines exhibit variable degrees of precipitation. In contrast, the PFKs from all the blood cells and lymphoblastoid cell line from the M-deficient patient exhibit total resistance to the precipitation.

(2) Anti-L antibody. An excess of this antibody precipitates completely the isolated L4 isozyme, however, muscle PFK (M4) is completely resistant. The PFKs from all the blood cells and cultured cell lines, both normal and M-deficient, exhibit variable but significant degrees of precipitation; the cultured
Fig. 2. Chromatographic separation of PFK isozymes from erythrocytes and cultured cell lines, showing multiple (more than 5) species in erythrocytes, cultured fibroblasts, and lymphoblastoid cell lines derived from normal individuals, but only 5 species in the lymphoblastoid cell line derived from the M-deficient individual.

The proposed multigenic control of PFK can only be conclusively established by (A) demonstration of the theoretically expected 1, 5, or 15 isozymic species in various tissues when 1, 2, or 3 of these genes are considered to be expressed; (B) production of the theoretically expected number of species, when one or more of these subunits are hybridized in vitro; (C) demonstration of the independent expression of two distinct subunits in the face of a genetic deficiency of the third subunit; and (D) demonstration of 3 distinct subunits by direct biochemical means, i.e., by comparison of their tryptic peptide maps and amino acid compositions and sequences.

While the immunologic data presented by Layzer et al. and Meienhofer et al. provided only indirect evidence as to the probable type(s) of subunit(s) present in a given cell type (since significant cross-reactivity was exhibited by their antisera), these could not clarify the number of isozymes, their precise subunit structures, and the relative proportions of various types of subunits contained therein. In contrast, in this study, we have utilized four distinct investigative approaches that have provided more direct evidence. These are as follows: (A) a high-

lymphoblastoid cell line from the patient exhibits nearly complete (100%) precipitation, as do the PFKs from his RBCs and WBCs. The platelet PFK from the patient exhibits approximately 70% precipitation, as do normal platelets (data not listed).

**DISCUSSION**

Based on immunologic evidence, Layzer et al. first proposed the existence of multiple isozymes of PFK in man. They considered the RBC and fibroblast PFKs to consist of single and multiple isozymes, respectively, composed of both muscle and nonmuscle type subunits. WBC and platelet PFKs were considered to lack M subunits, and each was believed to consist of at least two isozymes composed of the "nonmuscle" subunit and a third type of PFK subunit. Thus, at least three loci coding for various nonidentical subunits of PFK were proposed; an extremely insightful hypothesis, for which the present studies provide conclusive evidence. Recently, Meienhofer et al. have also suggested the existence of M (muscle), L (liver), and F (fibroblast) types of PFK subunits in various human blood cells based on differential reactivities of these PFKs to absorbed rabbit antisera.
resolution column chromatography technique to demonstrate directly the presence of multiple PFK isozymes in any given tissue; (B) protein hybridization techniques to confirm the subunit structure(s) of a given PFK inferred from the chromatography data; (C) studies of a patient known to be homozygous-deficient for M subunits to demonstrate the independent expression of the other two subunits; and (D) immunochemical analysis using exquisitely subunit-specific monoclonal antibodies to confirm conclusively the molecular structures derived from chromatographic and hybridization data.

To determine whether the deficiency of M subunits was quantitatively expressed in the tissues of our patient with Tarui's disease, we compared the PFK activity values of his various cell types with those of normal individuals (Table I). It is noteworthy that all types of nucleated cells exhibit much higher PFK activity levels than non-nucleated cells, i.e., RBCs and platelets. In sharp contrast, extremely high activity values are found in cultured fibroblasts, lymphoblastoid cell lines, and amnion cells. The strikingly elevated PFK activity exhibited by cultured cell lines probably reflects their heightened needs for energy and/or glycolytic intermediates for rapid cell replication. For instance, dormant lymphocytes exhibit approximately 10 U/10^10 cells; however, when transformed by Epstein-Barr virus into metabolically active, rapidly replicating lymphoblasts, they exhibit almost a tenfold increment in PFK activity. The lymphoblastoid cell line from the patient exhibits a quantitative PFK deficiency as do his RBCs.

The neutrophils and lymphocytes exhibit predominantly L4-type isozyme as suggested earlier; however, both cell types also exhibit the hybrid isozymes, composed of M, L, and probably P subunit in moderate amounts. The presence of the P subunit is inferred indirectly, since non-L4 isozymes account for approximately 40%-50% of the PFK activity chromatographically and yet only 20%-25% is precipitable by anti-M antibody. The reported absence of partial deficiency of PFK in our patient's WBCs may now be attributed to the fact that both cell types express only minor amounts of M subunits (~20%-25% M-containing species). Such a small reduction, if present, is not clearly apparent during quantitative assays.

The platelet PFK elutes as three species that occupy the general positions of RBC hybrids but are distin-
cultured cell lines, were observed. Five of these species and the formation of L4 and L3P subunits. The alterations in the relative proportions of various species, indicating a higher ratio of P:L, are indistinguishable from those of RBCs (M+). Upon dissociation and reassociation, platelet PFK indeed produced the theoretically expected five-membered set. The first three species are chromatographically indistinguishable from the original platelet isoforms and the last one from L4 isozyme, with an additional species (PL1) eluting just prior to L4 (Fig. 3A). The native platelet PFK exhibits a preponderance of P-containing species both chromatographically and immunologically, indicating a higher ratio of P:L subunits. The alterations in the relative proportions of various species and the formation of L4 and L1,P probably represent a selective loss of P subunits at acid pH with a more equitable P:L ratio during reassociation. The incomplete precipitation of platelet PFK (∼70%) with an excess of anti-L antibody is in accord with these data and indicates that anti-L does not precipitate P4 isoform.

To determine whether RBC PFK shows any quantitative or qualitative alterations in the relative proportions and types of isoforms, we surveyed RBC PFK of 13 individuals with different ethnic backgrounds. The previously reported five-membered isoform set was found in nine individuals with only minor variations in the relative proportions of various species. However, in four individuals (two of whom were siblings), 10–12 species, indistinguishable from those present in cultured cell lines, were observed. Five of these species are indistinguishable from those of RBCs (M+L), and three are indistinguishable from those of platelets (P+L), suggesting that all three types of subunits are present in these cells. This hypothesis is confirmed by the hybridization of RBC PFK (M+L) with platelet PFK (P+L), which yields the same 10–12 PFK species (Fig. 3B) as those observed in the fibroblasts. As illustrated in Fig. 3D, the random polymerization of three distinct subunits is theoretically expected to produce 15 different tetrameric isoforms: 3 homotetramers, 9 heterotetramers composed of 2 types of subunits, and 3 heterotetramers composed of all 3 types of subunits. The fact that chromatographically it is possible to resolve only 10–12 species, instead of the theoretically expected 15, reflects the unavoidable overlap of some of the species (Fig. 3D). The single peak observed between M4 and P4 probably represents the sum of three hybrid species composed of M and P, since M4 and P4 elute so very close to each other. The two heterotetramers composed of all three subunits probably cochromatograph as a single peak, thus yielding 10–12 chromatographically distinguishable species.

Normal lymphocytes predominantly express the L-containing isozymes; however, after Epstein-Barr-virus-induced malignant transformation in vitro, they express an increase in M- and P-containing species, suggesting increased synthesis of these subunits. Approximately 50% of the PFK from transformed lymphocytes is precipitable by anti-M as compared to 25% from normal lymphocytes. In contrast, the lymphoblastoid cell line from our patient exhibits only five species composed of P and L subunits, indicating the total absence of M subunits. These data further confirm our interpretations that the expression of 10–12 isozymes in some cell types is due to the simultaneous expression of 3 genes, since expression of only 2 genes in an M-deficient cell line results in the formation of the theoretically expected 5 species.

Our immunoprecipitation results closely corroborate the data derived from the chromatographic and molecular hybridization studies. As anticipated, an excess of anti-M precipitates approximately 80% of RBC PFK, since it precipitates not only M4, but also the hybrids of M with L. (Our previously reported value of 50% was probably due to the lack of excess antibody in the assay system, secondary to the low titer of the rabbit antibody.) Accordingly, anti-L precipitates also approximately 90% of RBC PFK. Platelet PFK is completely resistant to anti-M, even in very large excess, and is only partially precipitated (∼70%) with anti-L. In contrast, lymphocytes and neutrophils exhibit approximately 20–25% precipitation with anti-M and 80–90% with anti-L. All types of normal cultured cell lines, except amnion cells, exhibit approximately 50% precipitation with anti-M and 70% with anti-L. The cultured amnion cells exhibit approximately 20% and 50% precipitation with anti-M and anti-L, respectively. In contrast, all the blood cells as well as the cultured lymphoblastoid cell line from the patient exhibit total resistance to anti-M, indicating a complete absence of catalytically active "M" subunits. This may result either from a total synthetic failure or from the production of a catalytically silent gene product.

Based on these chromatographic and immunologic data, the "P" subunit is distinct from the "M" subunit. The definitive evidence for an independent
PFK ISOZYMES IN HUMAN BLOOD CELLS

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 genetic control for the “P” subunit is provided by the
 studies of the platelet PFK from the M-deficient
 patient, which exhibits a normal activity level and
 isozyme profile. The third subunit of PFK besides M
 and L is most appropriately called “P” and not “F,” as
 it is found more abundantly in platelets than in fibro-
blasts. Moreover, the letter “F” was initially utilized by
 other investigators to designate “fetal” isozyme and
 should appropriately be reserved for that usage only.28

 Of great potential significance is our observation that all three subunits are expressed in cultured fibro-
blasts (both adult and fetal) and amnion cells. This
 may prove to be useful in prenatal diagnosis of glyco-
genesis type VII, since the absence of M subunits
 would be detectable in fetal fibroblasts and/or amnion
 cells. This may also permit a detailed genetic dissec-
tion of the PFK isozyme system, since both inter- and
 intraspecific somatic cell hybrids may express all three
 normal as well as mutant PFK genes.

 Since PFK controls the rate of glycolysis in neoplas-
tic tissue as well, the increased synthesis of M and P
 subunits in lymphocytes after virus-induced blast
 transformation in vitro is of special interest. We
 recently surveyed various types of leukemic cells to
 establish whether similar changes in gene expression
 occur after malignant transformation in vivo. Our
 preliminary data suggest that leukemic cells exhibit a
 similar although less striking increase in synthesis of
 M and P subunits, but more importantly, exhibit
 striking increases in the total PFK activities. Thus,
 PFK is a transformation-linked discriminant of malig-
nancy.29,30

 In summary, these studies directly demonstrate the
 existence of multiple isozymes of PFK in normal and
 M-deficient human blood cells and cultured cell lines
 and provide direct molecular and genetic evidence as
 to their subunit structures. Three structural loci are
 shown to code for three distinct PFK subunits: M
 (muscle), L (liver), and P (platelet) types. Random
 polymerization of one or more of these produces one
 or more tetrameric isozymes. In any given tissue, the
 expression of 1, 2, or 3 genes leads to the formation of
 a maximum of 1, 5, or 15 tetrameric isozymes. The
 number and relative proportions of various species are
 determined by the relative rates of synthesis of
 different subunits. The number of species detected is
 also determined by the ability of the techniques used
to resolve and/or identify these extremely similar
 molecules.

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Isozymes of human phosphofructokinase in blood cells and cultured cell lines: molecular and genetic evidence for a trigenic system

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