Production and Characterization of Monoclonal Antibodies to the Subunits of Human Phosphofructokinase: New Tools for the Immunochemical and Genetic Analyses of Isozymes

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Recently we have demonstrated that human phosphofructokinase (PFK; ATP: D-fructose-6-P, 1-phosphotransferase; EC 2.7.1.11) is under the control of three structural loci that code for M (muscle-type), L (liver-type), and P (platelet-type) subunits; random tetramerization of these subunits produces various isozymes. In this study, we have produced and characterized BALB/c hybridoma antibodies to the M- and L-type subunits of human PFK. The specific antibodies were detected by an enzyme-immunoprecipitation assay using Staphylococci-bearing protein A as an immunoadsorbent. Of the wells tested using red blood cell (RBC) PFK (M + L), 61% were positive. Only one M-specific hybridoma was identified. The one anti-M and 4 anti-L antibodies were characterized for their biochemical and immunochemical specificities. To define the combining specificities of these antibodies, we compared their reactivity with and that of monospecific rabbit anti-M antiserum with muscle and liver PFKs from 15 different vertebrate species. The rabbit anti-M shows strong cross-reactivity with the muscle PFKs from all the species studied. In contrast, the monoclonal anti-M reacts exclusively with muscle PFKs from primates. Two of four anti-L antibodies react only with human L-PFK, whereas the other two react with that from a few other vertebrate species as well. Taken together, these data suggest that primate-specific antibodies recognize evolutionarily, recently acquired antigenic determinants, whereas the antibodies reactive with PFKs from distantly related species recognize conserved determinants. The differential immunoreactivities of muscle and liver PFKs strongly suggest the presence of distinct isozymes in all the vertebrate species studied. These studies demonstrate that it is feasible to produce and characterize monoclonal antibodies that distinguish among isozymes with structural and functional similarities. These antibodies provide sensitive tools in the analyses of isozyme structure, genetics, and related fields.

PHOSPHOFRUCTOKINASE (PFK; ATP: D-fructose-6-P, 1-phosphotransferase, EC 2.7.1.11), the key regulatory enzyme of glycolysis, is a tetrameric protein, with a molecular weight of approximately 340,000.1 Recent biochemical, immunochemical, and genetic studies from our laboratory have conclusively demonstrated that three structural loci code for distinct PFK subunits, i.e., M (muscle-type), L (liver-type), and P (platelet-type).2-4 A tissue may express one, two, or all three genes; the products of these genes randomly form tetramers, some of which are clearly separable by ion-exchange chromatography. However, as chromatography does not distinguish between all isozymes, we decided to undertake the immunochemical analysis of various human PFKs using subunit-specific monoclonal antibodies.

This article describes the production and characterization of hybridoma antibodies specific for muscle- and liver-type human PFK isozymes. Five monoclonal antibodies, one specific for M-subunit and four specific for L-subunit are characterized. The reactivity patterns of the monoclonal antibodies with PFKs from a number of vertebrate species showed that the four L-specific hybridomas recognized different antigenic determinants. These reactivity patterns, combined with that of the monospecific rabbit anti-M antiserum, strongly suggest the existence of isozymes of PFK in all the vertebrate species studied, as well as structural homologies among various vertebrate PFKs.

Recently we have successfully utilized the subunit- and human-specific antibodies to aid in the immunochemical and genetic analyses of the human PFK isozyme system.4-6 Our results as a whole indicate that monoclonal antibodies of defined specificities provide sensitive and versatile tools in the analyses of isozyme structure, genetics, and related fields. Preliminary results of these studies have been presented.7

MATERIALS AND METHODS

Reagents and culture media. Iscove’s modified Dulbecco’s medium (IMDM) and fetal calf serum (FCS) were products of Grand Island Biological, Grand Island, N.Y. Freund’s complete and incomplete adjuvants came from Difco Laboratories, Detroit, Mich. Polyethylene glycol (PEG-1000) was obtained from Baker, Phillipsburg, N.J., and Cowan strain of Staphylococci-bearing protein A (IgG sorb; binding capacity 2.0 mg IgG/ml of 105/v cell suspension) from the Enzyme Center, Inc., Boston, Mass.; 2,6,10,14-tetramethyl pentadecane (Pristane) was the product of Aldrich Chemicals, Milwaukee, Wisc. Nonidet P 40 (NP-40) was purchased from Sigma Chemical Co., St. Louis, Mo.

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from Particle Data, Elmhurst, Ill. Subclass-specific antisera were purchased from Meloy Laboratories Inc., Springfield, Va.

Animals and cell lines. BALB/c and CDF (F1 hybrids of BALB/c x DBA 2) mice of both sexes, 6 wk old, were obtained from West Seneca Farms, West Seneca, N.Y. The 45.6: TG.1.7 line, a BALB/c myeloma cell line resistant to 5 μg/ml of thioguanine and killed by hypoxanthine-aminopterin-thymidine (HAT) medium, was obtained from Matthew Scharff, Albert Einstein College of Medicine, Bronx, N.Y.; 45.6: TG.1.7.5 (clone 5), a subclone synthesizing only light chain (k) was isolated in our laboratory.4 All cell lines were maintained in IMDM and 10% FCS or 5%–10% horse serum, containing penicillin (10 U/ml) and streptomycin (70 μg/ml), at 37°C in 5% CO2 and 95% air.

Phosphofructokinase assays were performed with a Gilford Model 2400 spectrophotometer at 26°C, as described previously.2

Production of the rabbit anti-M antibody has been described previously.2

Purification of various PFKs. The human muscle, RBC, and liver PFKs were partially purified according to the methods described previously.2 Since the liver also contains hybrid isozymes (~40%), pure L3 homotetramer was obtained after DEAE-cellulose chromatography as described by Brock.9 The pure P1 homotetramer was obtained by DEAE-Sephadex A-25 chromatography of platelet PFK as described earlier.4

Immunization procedure. Twenty units of partially purified RBC PFK (approximately 200 μg of enzyme protein) were diluted with normal saline (0.15 M NaCl) to a final volume of 0.5 ml and emulsified with an equal volume of Freund's complete adjuvant. For the primary immunization, the BALB/c (female, 6 wk old) mouse received 50 μl of this emulsion (20 μg enzyme) in each of the hind footpads. Booster immunizations of 20 μg of antigen in incomplete Freund's adjuvants were given at 3- and 6-wk intervals subcutaneously over the abdomen.

Cell fusion and cloning. Myeloma cells (clone 5) and spleen cells from the mice showing the highest antibody titers against RBC PFK (M + L) were fused using 35% PEG-1000 as previously described.10 Approximately 15 hr later, the cells were resuspended in IMDM + 20% FCS + HAT and seeded into individual wells (4 x 105 spleen cells/well) of microtiter plates (96 wells). When the hybrid cells were semicolumnal, the supernatant was tested for the presence of antibodies by the enzyme immunoprecipitation assay, using either RBC PFK or muscle and liver PFKs as antigens. Cells from positive wells were cloned in soft agarose,10 clones randomly recovered, and screened for the specificity of their reactivity.

Screening of hybrids using enzyme immunoprecipitation. Staphylococci-bearing protein A (IgGsorb) were used to precipitate soluble antigen–antibody complexes formed by hybridoma antibodies, as described previously.45 Fifty microliters of PFK (diluted to 0.05–0.1 U/ml in 50 mM potassium fluoride buffer, pH 7.5, containing 7mM (NH4)2SO4, 0.7 mM EDTA, 3 mM dithiothreitol, 1 mM L-α-amino-capric acid, 0.7 mM AMP, and 0.7 mM Fru-6-P) was mixed with 50 μl of the hybridoma supernatant to be tested; control tubes contained either culture medium or supernatants from wells in which no hybridoma, but fibroblast growth was present. The mixtures were incubated for 30 min at 37°C and then 100 μl of a freshly washed 10% suspension of IgGsorb was added.14 The mixtures were incubated at 4°C for 30 min with continuous shaking, centrifuged at 4000 g for 10 min, and assayed for residual enzyme activity. Only the supernatants precipitating more than 10% of PFK as compared to the controls were considered positive.

Analysis of hybridoma proteins. The class and subclass of the hybridoma proteins were determined by Ouchterlony analysis using subclass-specific antisera.3 To obtain radiolabeled Igs, the hybridoma cells (104) were washed twice in Eagle's spinner medium,12 depleted of methionine, and supplemented with nonessential amino acids and glutamine. They were resuspended at a concentration of 105 cells/ml and incubated for 15 hr in the above medium plus 2% FCS to which 35S-methionine was added to a final concentration of 1.7 μCi/ml. The radiolabeled antibodies were immunoprecipitated from the supernatants using specific rabbit antibodies and IgGsorb. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels in Tris-HCl buffer.11 Autoradiography was performed using Kodak SB-5 x-ray film.

Production of ascites in mice. Male CDF1 mice (6–8 wk old) were injected with 0.2 ml of Pristane intraperitoneally, followed by 1–2 x 105 hybridoma cells after 2–4 wk. Ascites fluids were produced within 12–14 days and were collected every second or third day until the death of the mouse.

Immunoprecipitation of vertebrate PFKs. Specimens of muscle and liver tissues from the following vertebrate species were obtained either commercially or from various investigators at the College of Physicians and Surgeons, Columbia University, New York, N.Y.: human, monkey (pig-tail), dog, sheep, cattle, pig, rabbit, mouse (CDF, and BALB/c), Chinese hamster, chicken, turkey, turtle, and fish. Small pieces of tissue were washed extensively with chilled phosphate-buffered saline to eliminate as much blood as possible. Crude extracts prepared using the potassium fluoride buffer described earlier were tested by immunoprecipitation using a 1:3 dilution of rabbit anti-M, and 1:200 dilutions each of the ascites fluids containing the four monoclonal antibodies (V44-08, V47-20, V65-06, and V96-26) and the culture supernatant of one hybridoma (V44-09).

RESULTS

Hybridization

Of 10 injected mice, those four showing the highest serum titer (by immunoprecipitation) were given an additional injection of RBC PFK. On the fourth day

Fig. 1. Screening of hybridoma supernatants for specific antibodies by enzyme-immunoprecipitation assay.
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Virtually every well showed growth of hybridomas with multiple visible clones. The hybridoma supernatants were tested for the specific antibodies by using partially purified RBC (M + L), muscle (M4), and liver (L4) type PFK isozymes as antigens in the enzyme-immunoprecipitation assay. The flow chart shown in Fig. 1 illustrates the sequence of studies that were undertaken and the results obtained during the screening procedure. A total of 537 hybrid supernatants were tested for antibody activity; in the initial screen using RBC PFK (M + L), 62 of 102 supernatants tested, or 61%, gave a positive reaction. The remaining 435 supernatants were screened only for the reactivity with muscle PFK; none was found to be positive. The 15 supernatants showing 50%-92% precipitation were restested against muscle and liver PFK separately; 14 reacted with liver PFK only, while one reacted with muscle enzyme. Cells from these wells were cloned in soft agarose, and reactive clones recovered from four of the wells (Table 1). The two clones recovered from well V44 differ as to their Ig subclass (γ1 and γ2a), whereas the two clones from V96 do not (γ1). Using an SDS-PAGE system designed to distinguish between different light chains, the clones from V44 were shown also to possess different light chains, but not those from V96 (Fig. 2). All the clones exhibit the same subunit specificity as that of the parent wells. All of the six hybridomas produce subunit-specific antibodies: four with anti-L (V44-08, V44-09, V47-20, and V65-06) and two with anti-M (V96-26 and V96-55) reactivities (Table 1). All data suggest that V96-26 and V96-55 resulted from the same fusion event.

Table 1. Specificity and Ig Subclass of the Hybridomas

<table>
<thead>
<tr>
<th>Well</th>
<th>Subclones</th>
<th>Specificity*</th>
<th>Subclass</th>
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<tbody>
<tr>
<td>V44</td>
<td>V44-08</td>
<td>L</td>
<td>γ1</td>
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<td></td>
<td>V44-09</td>
<td>L</td>
<td>γ2a</td>
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<td></td>
<td>V96-55</td>
<td>M</td>
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*All were tested against homotetramers of human PFK purified by methods described in the text.

Fig. 2. Autoradiography of SDS-polyacrylamide gels (12.5%) showing biosynthetically labeled reduced hybridoma proteins. Migration is from top to bottom.

Immunoprecipitation of Vertebrate PFKs

To define the antigenic specificities of the monoclonal antibodies, we compared the immunoprecipitation values of PFKs from 15 vertebrate species. Table 2 lists the percent precipitation values of the PFKs from the five reactive species only. The monoclonal antibodies did not react significantly with PFKs from any of the other species. Undiluted rabbit anti-M exhibits significant cross-reactivity with human L4 and P4 homotetramers in the immunoprecipitation assay; however, when diluted 1:3 it reacts only with human M4 (Table 2). The diluted rabbit anti-M cross-reacts strongly with the muscle PFKs from all the species (mean precipitation 92% ± 11%), except those from chicken, turkey, and fish (mean precipitation 39% ± 15%). In contrast, it reacts relatively less with liver PFKs from most of the species (mean precipitation 44% ± 12%), except those from dog, rat, chicken, turtle, and fish (mean precipitation 10% ± 11%). The five monoclonal antibodies (1 anti-M and 4 anti-L) showed reactivity only with the PFKs from the five species listed in Table 2. V96-26, the only anti-M, is found to react with primate M-PFK, two of the four anti-L antibodies (V44-09 and V47-20) react only with human L-PFK, whereas the other two anti-L
antibodies react variably with L-PFKs from other species.

**DISCUSSION**

In the past, several investigators have utilized rabbit antisera against partially purified human muscle PFK to undertake immunochemical analysis of the then putative isozymes of PFK in man. In 1967, based on differential reactivities of human muscle and red cell PFKs to the rabbit anti-M antiserum, Tarui and Layzer et al. first proposed the existence of two nonidentical subunits of PFK in man. In 1970, based on differential reactivities of PFKs from various human cell types, Layzer et al. first proposed the existence of multiple isozymes in man, consisting of at least three types of subunits. Thus, at least three loci coding for various nonidentical subunits of PFK were proposed for which the recent biochemical, immunochemical, and genetic studies from our laboratory have provided conclusive evidence. Recently, Kahn's group has also suggested the existence of three distinct subunits of PFK based on differential reactivities of various human PFKs to three types of absorbed rabbit antisera.

Initially we attempted to raise monospecific antibodies to the three human PFK subunits in the rabbit. The production of a rabbit anti-M antibody was accomplished with relative ease, because the homotetramer M₄ can be isolated in milligrams quantities from readily available fresh muscle tissue. In contrast, the production of monospecific anti-P and anti-L antibodies was fraught with numerous problems because of the following two reasons. First, no human tissue contains only P₄ or L₄ isozyme, and second, these homotetramers are difficult to purify in sufficient quantities from tissues containing a mixture of isozymes. Our rabbit anti-P and anti-L antisera produced by using homogeneous preparations of brain and placental PFKs continued to exhibit cross-reactivities despite extensive absorptions. Similar experience has been reported by other investigators.

Therefore, we decided to use the hybridoma technique to produce monospecific antibodies. Recently Slaughter et al. have produced monoclonal antibodies to human placental alkaline phosphatase isozyme that detect polymorphic variation at one and possibly two of the determinant sites.

In the present study, a total of only 3–5 U of RBC PFK (30–50 µg enzyme protein) per mouse proved to be a good immunogen, since all the mice exhibited serum antibodies. Since monoclonal antibodies are often nonprecipitating, we used *Staphylococci*-bearing protein A to precipitate soluble antigen–antibody complexes and measured the supernatants for residual enzyme activity. The immunoadsorbent was added in an amount (100 µl of a 10% suspension) whose binding capacity (~200 µg IgG) exceeded the approximately 100 µg of IgG present in the 50 µl of culture supernatant (10% FCS plus secreted antibodies). However, the possibility that some positive wells may have been missed, if they contained antibodies either in amounts too small to precipitate more than 10% of the test enzyme or an Ig that does not bind *Staphylococcal* protein A, cannot be excluded. The enzyme-immunoprecipitation using IgGsorb appears to be an
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extremely simple, rapid, sensitive, and reproducible screening procedure.

The first 102 wells tested against RBC PFK showed a rather high frequency (61%) of positives. However, the overwhelming majority of these hybridomas showed anti-L reactivity, despite the fact that the immunogen RBC PFK consists of M and L subunits in approximately equal proportions. The sera of the immunized mice also showed almost exclusively anti-L antibodies. These data suggest that the human L subunit is immunodominant in mice, probably secondary to the fact that it is structurally less related to the mouse PFKs than to human M subunit. However, anti-M antibodies are readily produced when muscle PFK is either injected alone or in combination with P subunit. As shown in Table 1, when the contents of some of the positive wells are cloned, the subclones derived from a single well exhibit the same subunit specificities. The two clones derived from the well V44 differ not only in the IgG subclass (Table 1) but also in the light chain (Fig. 2), indicating that these are products of independent fusion events. In contrast, the two clones arising from the well V96 exhibit identical heavy chain subclass, light chain, and subunit specificity and are therefore considered to be identical. All of these antibodies are subunit specific; V96-26 and V96-55 react exclusively with the human L subunit, and V44-08, V44-09, V47-20, and V65-06 react exclusively with the human L subunit even when used in excess (4–18-fold), indicating that these react with antigenic determinants unique to each human subunit. In addition, all these antibodies maintain their respective subunit specificity when they exhibit reactivities with nonhuman PFKs, i.e., all anti-L antibodies only react with liver PFKs from other vertebrate species (Table 2).

The failure to find any subunit cross-reactive antibodies may be more apparent than real, since we characterized in detail only a small sample of monoclonal antibodies from the entire repertoire of the mouse. Alternatively, it is possible that cross-reactive antibodies may not have been generated at all by the mouse, since such antibodies may be directed against the antigenic determinants that are shared among isozymes or species. The shared determinants may represent highly conserved parts of the enzyme molecule, e.g., catalytic sites or subunit–subunit interaction sites where conservation of protein structure is essential for both enzyme activity and the ability to form heteropolymers. Since antibodies directed against such antigenic determinants may constitute autoantibodies, their production may be completely excluded by the mouse. Therefore, monoclonal antibodies may by necessity be directed against parts of the PFK molecule that can tolerate amino acid substitution without loss of function.

To define the antigenic specificities of various monoclonal antibodies and the evolutionary interrelationships among vertebrate PFKs, we compared the immunoprecipitation values of muscle and liver PFKs from 15 different vertebrate species (encompassing all five orders) using both the rabbit heteroantiserum and five monoclonal antibodies; four anti-L and one anti-M. The homologous PFKs provide an array of antigenic determinants, which may or may not be shared among various vertebrate species. The identity or nonidentity of reactivity pattern of any two monoclonal antibodies can then establish the identity or nonidentity of their antigenic specificities. Conversely, such an analysis using a panel of monoclonal antibodies can discriminate between the identity or nonidentity of antigenic determinants of any two PFKs, either from the same or different species. The degree of immunochemical relatedness within a set of homologous proteins reflects the degree of homology of their primary structures, and therefore their evolutionary interrelationships. This strategy of “reactivity pattern analysis” has been employed by various investigators to either define structurally similar proteins and enzymes, their antigenic determinants, and the phylogeny of either the proteins or their determinants or to characterize monoclonal antibodies.

From the data presented in the Results section and Table 2, the following conclusions can be reached. All of the muscle PFKs studied exhibit strong cross-reactivities with the rabbit anti-M antibody, suggesting the presence of structural homology among these enzymes, which indicates the highly conserved nature of the vertebrate muscle PFK isozyme. As anticipated, the degree of cross-reactivity is lesser for the PFKs from lower vertebrates as compared to the higher vertebrates. The partial reactivity of various liver PFKs with rabbit anti-M is most likely due to the presence of both muscle-type and non-muscle-type isozyme(s) in different hepatic cell types. Present data on liver PFK isozymes in man (Table 2) and in the rat support such a conclusion.

Three of the five monoclonal antibodies react only with primate PFKs. The anti-M antibody (V96-26) reacts with the muscle-type PFKs from human and monkey only, whereas anti-L antibodies (V44-09 and V47-20) react with human L4 only. These three antibodies probably recognize antigenic determinants recently acquired during evolution and suggest a high degree of structural homology between human and monkey PFKs, which is consistent with data from other proteins and their known phylogeny in relation to man. In contrast, two anti-L antibodies (V44-08...
and V65-06) react variably with liver-type PFKs from the monkey, dog, guinea pig, and turtle. The differential reactivity patterns of these two antibodies with the four liver PFKs provide evidence for their distinct combining specificities. In contrast, in the absence of such evidence, the two human-specific anti-L antibodies may very well represent identical combining specificities. It is of interest to note that each antibody maintains its subunit specificity across the species barrier; anti-M and anti-L antibodies react exclusively with muscle and liver PFKs, respectively. The immunological studies of rodent PFKs by Gonzalez et al., utilizing heteroantisera against rabbit PFKs (which were not monospecific since their anti-M antiserum precipitated 25% of rabbit L4 isozyme) have also suggested greater similarities among rodent muscle isozymes or liver isozymes than those between the muscle and liver isozymes of the same species. Taken together, these data suggest that to a variable extent, the protein structure of the vertebrate L subunit has also been conserved during evolution.

Although the isozymic nature of PFK is well established only in the case of man2,4,14,20 and the rodents,3,5,18,38 it seems plausible that PFK may exist in isozymic forms in other species as well. The differential immunoreactivities of PFKs from the muscle and liver of various vertebrate species, both with the rabbit anti-M and with the panel of anti-L monoclonal antibodies, suggest the presence of distinct isozymes in these two tissues. Since such differences, and therefore distinct isozymes, appear to be present in the turtle, the duplication of PFK genes and subsequent divergence appear to be ancient. Despite this early divergence, there seems to have been conservation of protein structure, probably necessary both to preserve enzyme activity and the ability to make active heteropolymers.34 In addition to the immunoechemical evidence, homology (both interspecific and intraspecific) among the vertebrate PFKs is indicated by the similarities of the physicochemical and kinetic properties among various vertebrate PFKs1 and the structural similarities among the chicken, rabbit, and human PFKs.39-41 Using a single monoclonal antibody, their degree of relatedness is not obvious, because each antibody possesses a single combining specificity and therefore generally gives an all-or-none reaction with any single PFK. Therefore, to undertake phylogenetic studies, it is necessary to use a large panel of monoclonal antibodies of differing specificities in order to simulate the results of heteroantisera.

The uses of isolated monoclonal antibodies with defined specificities are legion. Recently, we have successfully exploited the subunit specificity of these antibodies to undertake the precise immunoechemical analysis of human PFK isozymes from blood cells and cultured cell lines4 and their species-specificity to discriminate between human and Chinese hamster PFKs in the interspecific somatic cell hybrids to assign human PFKM and PFKL loci to chromosomes 1 (region p32 → q32) and 21, respectively.3,6

Our results show that it is possible to produce and characterize monoclonal antibodies with unique specificities to structurally and functionally similar isozymes. Such antibodies serve as powerful tools in the analyses of such complex isozyme systems. It is anticipated that the methods of production and characterization of monoclonal antibodies described in this paper and their applications described elsewhere will prove useful in similar analyses of other isozyme systems.

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Production and characterization of monoclonal antibodies to the subunits of human phosphofructokinase: new tools for the immunochemical and genetic analyses of isozymes

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