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Analysis of phosphofructokinase subunits and isozymes in ascites tumor cells and its original tissue, murine mammary gland

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Abstract Phosphofructokinase (PFK) subunits and isozymes have been examined in ascites tumor cells and murine mammary gland, the tissue from where this tumor originated. Ascites tumor was found to contain predominantly the C-type subunit, whereas the L-type subunit was more abundant in mammary gland. An altered M-type subunit of lower electrophoretic mobility was found in both cell types and no M_4 homotetramers were detected in either of them. Characteristic regulatory properties of ascites tumor PFK, i.e. insensitivity to fructose-1,6-P_2 activation and inhibition by P-enolpyruvate, were also observed in the mammary gland isozyme. The nature of these properties and the contribution of the distinct subunit types to fructose-1,6-P_2 activation are discussed.

Key words: Phosphofructokinase; Fructose-1,6-bisphosphate; Glycolysis; Allosteric regulation; Ascites tumor cell; Mammary gland

1. Introduction

Phosphofructokinase (PFK) from mammalian cells is known to be composed of three types of subunits (C, M and L) that have different electrophoretic mobility and form homo- and hetero-oligomers [1–4]. This gives rise to a series of isozymes which smallest active species are tetramers. Each subunit is encoded by different genes that exhibit tissue-specific expression. Thus, adult muscle contains only the M-type subunit, liver mainly contains the L-type subunit and all three types are present in brain [1,2]. The general pattern of PFK as a highly modulated allosteric enzyme whose activity is controlled by a number of effectors [5] is common to the various isozymes. However, the subunit composition has been shown [2,6–10] to promote kinetic and regulatory differences, mostly quantitative, among the isozyme pools that affect to the affinity for fructose-6-P and for some of the effectors, such as ATP, AMP or fructose-2,6-P_2, and that were suggested [2,6,8,9] to contribute to the characteristics of the glycolytic operation in particular tissues.

In ascites tumor cells, PFK activity has been demonstrated [11] to strongly limit the glycolytic flux under certain conditions and some individual regulatory properties, such as a lack of activation by fructose-1,6-P_2, were reported [12] for this enzyme. PFK isozymes have been investigated in tumor cells [3,13–16], but no determinations of their subunit distribution were described. In the present work, we have analyzed the subunit composition and isozymes of ascites tumor PFK by electrophoretic, immunological and chromatographic techniques to gain some insight into the bases of its peculiar regulatory behavior. As the Erlich-Lettré strain of ascites tumor was obtained from a transplantable mouse carcinoma of mammary origin [17,18], we have also examined in a comparative way the subunits and properties of the enzyme from murine mammary gland to investigate whether the particular characteristics of PFK from ascites tumor are related to the malignant nature of these cells.

2. Materials and methods

2.1. Materials

Biochemicals were purchased from Boehringer-Mannheim or Sigma. Auxiliary enzymes, blue Sepharose CL-6B and protein-A–Sepharose CL-4B were obtained from Sigma. DEAE-trisacryl M was from Biotechnics. Goat anti-(rabbit immunoglobulin G)-peroxidase conjugate was from Nordic. Antibodies against the M and L subunits of rat muscle and liver PFK, respectively, and against brain PFK were kindly donated by Dr. G.A. Dunaway (Southern Illinois University).

2.2. Cells and tissues

A hypertriploid strain of Ehrlich-Lettré ascites carcinoma cells was grown in the abdominal cavity of 2-month-old male Swiss mice. Cells were harvested and washed as described previously [12]. Muscle and liver were obtained from male Swiss mice. Brain was obtained from male Sprague-Dawley rats. Mammary gland tissue was obtained from lactating Swiss mice on day 8–10 of lactation.

2.3. Preparation of extracts

Fresh cells and tissues were washed in 115 mM NaCl, 5 mM potassium phosphate, pH 7.4, and suspended in 2–3 vol. extraction buffer as described below. Ascites cells were frozen and thawed twice in liquid nitrogen. Tissues were homogenized in a Polytron. The homogenates were centrifuged at $9000 \times g$ for 10 min at 4°C and then at $105\,000 \times g$ for 30 min. The supernatant fluids were collected.

2.4. Analysis of PFK isozymes

PFK isozymes were analyzed by SDS-PAGE, Western blotting and immunotitration with specific antibodies and ion exchange chromatography (DEAE-trisacryl).

For SDS-PAGE, Western blotting and immunotitration, extracts in buffer A (50 mM Tris-phosphate, 0.05 mM fructose-1,6-P_2, 0.1 mM EDTA, 1 mM DTT, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, pH 8.0) were chromatographed on a column (1×5 cm) of blue Sepharose CL-6B equilibrated with buffer A. The column was washed with 50 ml of buffer A and then with the same buffer containing 0.15 mM ADP. PFK activity was eluted with buffer A containing 2 mM fructose-6-P and 2 mM ATP. Fractions of 2 ml were collected and those showing PFK activity were pooled. Complete recovery of activity was obtained. The pooled fractions were subjected to SDS-PAGE employing 6% polyacrylamide gels (17×22 cm) according to Laemmli [19]. The gels were stained with silver [20]. The percentage of each subunit was determined from silver-stained gels as relative to the band intensities quantified with a color image scanner (Seiko Epson G520A). The apparent M_r values of each subunit were determined by using the standards human plasma α_2-macroglobulin (180 000), *Escherichia coli* β-galactosidase (116 000), rabbit muscle phosphofructokinase (84 000), chicken muscle pyruvate kinase (58 000) and porcine heart fumarase (48 500). Transfer of the gel proteins to nitrocellulose paper and Western analysis of PFK subunits

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with each type of antibody were performed as described previously [21], except that the antibody was diluted 1:400. Immunotitrations of the pooled blue-Sepharose fractions with PFK antibodies were conducted as previously outlined [21], except that anti-IgG was mixed with partially purified PFK in the proportion 3:1.

Chromatographic analysis of PFK isozymes was carried out essentially as described by Etiemble et al. [22]. Extracts in buffer B (20 mM Tris-phosphate, 10 mM ammonium sulfate, 10 mM NaF, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 0.01 mM fructose-1,6-P_2, 0.2 mM ATP, pH 7.5) were applied to a column (1.9×7.8 cm) of DEAE-trisacryl equilibrated with the same buffer and 3-ml fractions were collected. PFK activity was eluted with a linear gradient of buffer B and the same one supplemented with 90 mM ammonium sulfate, 73.5 mM potassium phosphate and 50 mM NaCl.

2.5. Assay for PFK

Unless otherwise stated, the assay mixture contained 50 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, pH 7.2, 0.15 mM NADH, 5 mM Pi, 1 mM NH_4^+, 0.1 mM cAMP, 1 mM MgATP, 1.2 U aldolase, 10 U triosephosphate isomerase, 1 U glycerophosphate dehydrogenase and 5–10 μl of enzyme preparation in a total volume of 1 ml. After 5 min, the reaction was started by adding 1 mM fructose-6-P and was followed by measuring the absorbance change at 340 nm in a Perkin Elmer Lambda 5 spectrophotometer at 25°C. Glucose-6-P was always added to fructose-6-P in a proportion of 3:1. When PFK activity was measured under near V_{max} conditions, the assay was carried out at 0.5 mM MgATP, 5 mM fructose-6-P, pH 8.5. When the effects of fructose-1,6-P_2 and fructose-2,6-P_2 were examined, pyruvate kinase (1 U) and lactate dehydrogenase (1 U) were used as coupling enzymes in the presence of 0.12 mM P-enolpyruvate and the absence of Pi, NH_4^+ and cAMP; to minimize the inhibitory effect of P-enolpyruvate on PFK activity [12], its concentration in this assay was about 7 times lower than the obtained K_i value of 0.83 mM (see below). Auxiliary enzymes were desalted by centrifugation and dialysis against 10 mM HEPES, pH 7.0, 20% (v/v) glycerol. One unit (U) of activity is defined as the amount that catalyzes the conversion of 1 μmol substrate/min under these conditions. Protein was determined by the method of Bradford [23] with bovine γ-globulin as standard.

3. Results and discussion

3.1. Subunit composition and PFK isozymes

Ascites tumor cells exhibit a high PFK activity, which was found to be close to that measured in mouse muscle and rat brain and greater than that in mouse liver (18.6 ± 1.2, 27.0 ± 2.4, 12.3 ± 1.8 and 2.4 ± 0.15 U/g wet mass, respectively) when assayed in cell extracts under near V_{max} conditions. This agrees with the enhanced glycolytic activity reported in these [11] and other proliferating cells [24]. Analysis by SDS-PAGE and Western blotting with antibodies against L- and M-type subunits and against rat brain PFK, and subunit quantification from silver-stained gels (Fig. 1A), indicated that ascites tumor contains subunit types C (50%) and L (32%), with M_r values of 87 500 ± 600 and 78 700 ± 1000, respectively, as well as a lesser amount (18%) of an altered M-type subunit of lower electrophoretic mobility (M_r = 86 200 ± 600) than the M-type subunit of muscle (M_r = 84 000), that partially overlapped with the band of the C-type subunit and which we called M*-type. A similar difference between the M_r values of the subunit types M and M* was observed when ascites tumor PFK was compared with the isozyme from brain, where 49% of the subunits are M-type [1] (data not shown). Ascites tumor cells are therefore among those with the highest expression of the C-type subunit, such as rat intestinal mucosa, testis and spleen [1] and human fibroblasts [2]. An increase in C-rich isozymes was also reported in several malignant tissues [3,16], thus it appears that a high expression of this subunit type could be usual in tumor cells. The lower content of the M*-type subunit detected in ascites tumor was confirmed by immunotitration of the PFK activity, as increasing amounts of the antibody against the M-type subunit immu-

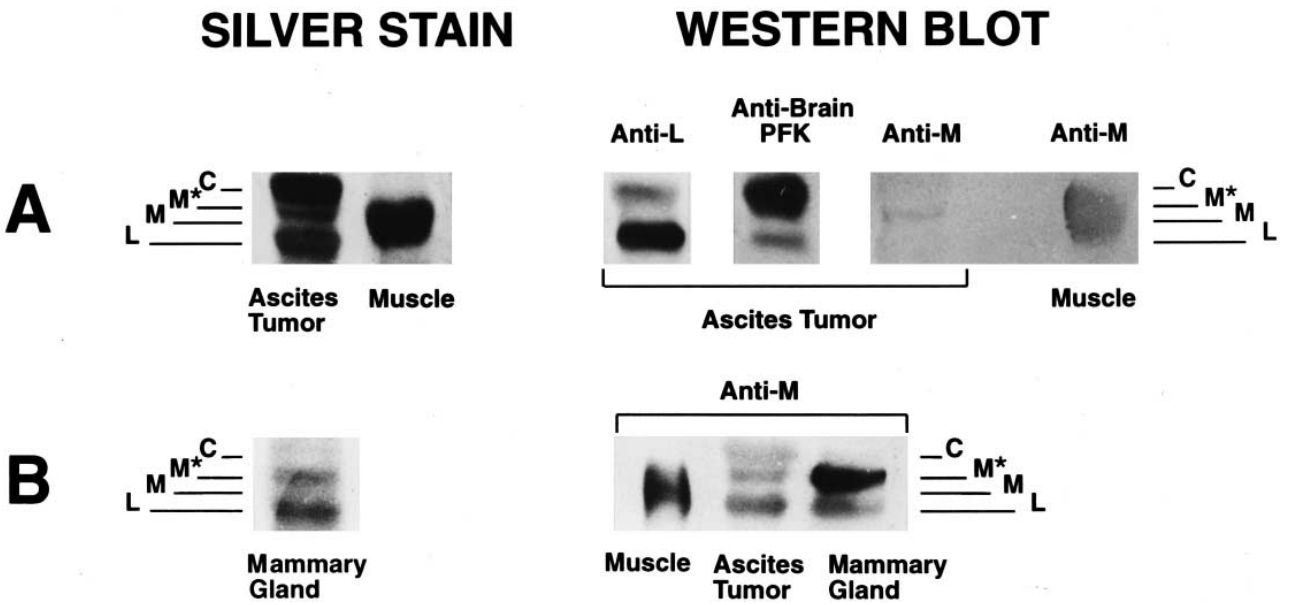

Fig. 1. Silver stain and Western blot analysis of PFK subunits in ascites tumor cells (A) and murine mammary gland (B). PFK isozymes were partially purified from each tissue by blue-Sepharose chromatography an subjected to SDS-PAGE as described under Section 2. Western blots were probed with antibodies as indicated. B: Samples from muscle and ascites tumor were also included for comparison with mammary gland; peroxidase reaction was allowed to proceed for a longer period of time than in (A) to improve visualization of the M*-type subunit in tumor cells.

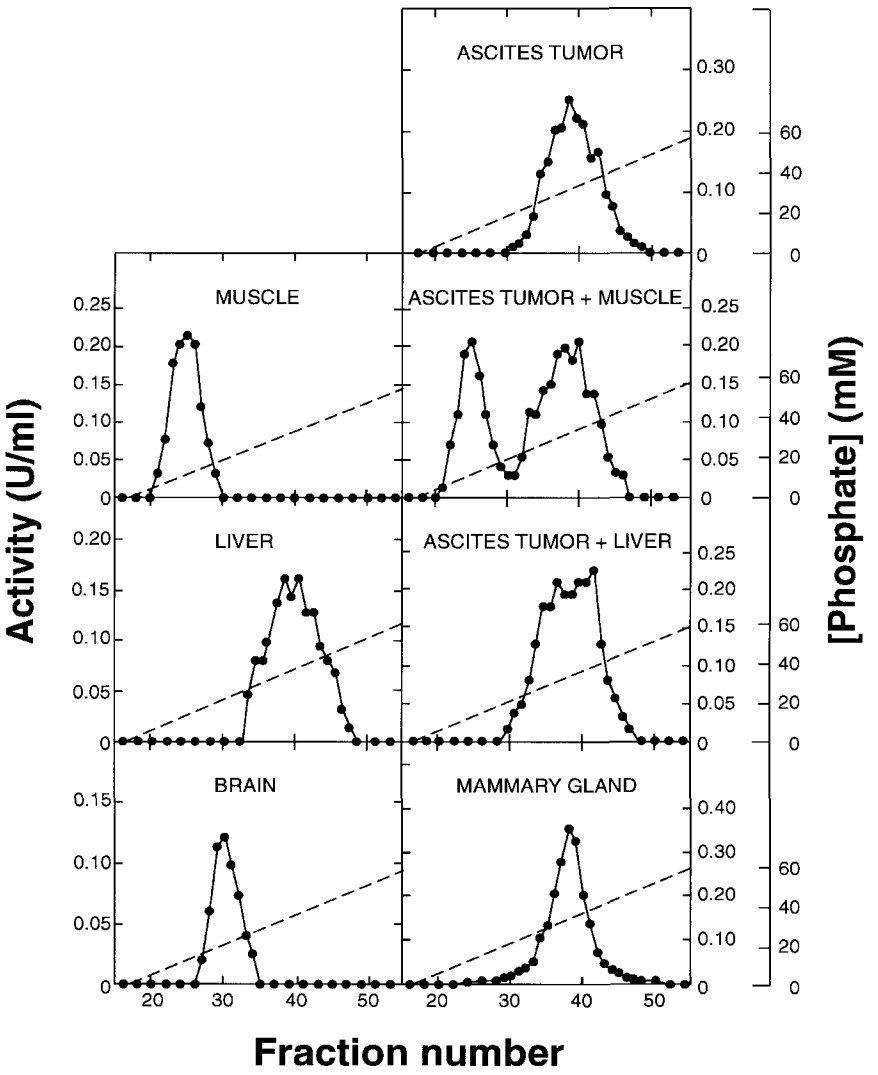

Fig. 2. DEAE-trisacryl chromatography of PFK isozymes from ascites tumor cells and various tissues of the mouse. High-speed supernatants were chromatographed on a DEAE-trisacryl column either separately or combined as indicated. Brain tissue was from rats. The method of elution was as described under Section 2.

noprecipitated 19% of the enzyme activity from the partially purified preparation of enzyme by blue-Sepharose, whereas it immunoprecipitated over 90% of the PFK activity in an enzyme preparation from skeletal muscle. As shown in Fig. 1B, the M*-type subunit was also found in murine mammary gland. This tissue contained the M*-type subunit in a higher proportion (33%) and consisted predominantly of the L-type subunit (65%), with only a 2% of the C-type subunit. We do not know at present whether the higher M_r value of the M*-type subunit, as compared to that of the M-type subunit, is related to some difference in their coding sequences or to a post-translational modification.

To gain some insight into the association of the different subunit types, PFK isozymes in ascites tumor cells as compared to those in muscle, liver, brain and mammary gland were analyzed by DEAE-trisacryl chromatography (Fig. 2), which was previously used by Etiemble et al. [22] for the separation of PFK isozymes in human erythrocytes. Although this method did not successfully resolve the various isozymes, since tumor PFK activity eluted as a broad heterogeneous peak, it clearly indicated that the early eluting homotetramers of the M_4 type are virtually absent in ascites tumor cells. This was best observed when a mixture of muscle (which contains only M_4 homotetramers [1]) and tumor cell extracts was chromatographed. This result agrees with data from Sumi and Ui [13] reporting greater affinity of ascites tumor PFK for DEAE-Sephadex than the muscle isozyme. As expected from the individual chromatographic profiles, the mixture of liver and ascites tumor exhibited an enrichment in late eluting species which are probably composed mostly of L-type subunits, the predominant subunit type in liver [1] and which Etiemble et al. [22] showed to have the highest affinity for DEAE-trisacryl. Interestingly, this analysis also indicated that M_4 homotetramers are practically absent in liver and mammary gland and are present in a very low proportion, if any, in brain, despite that this tissue contains a 49% of the M-type subunits [1], thus suggesting that as soon as L- or C-type subunits are present, the equilibrium of the M-type subunits is shifted to the formation of hybrids and practically no homo-oligomers are formed.

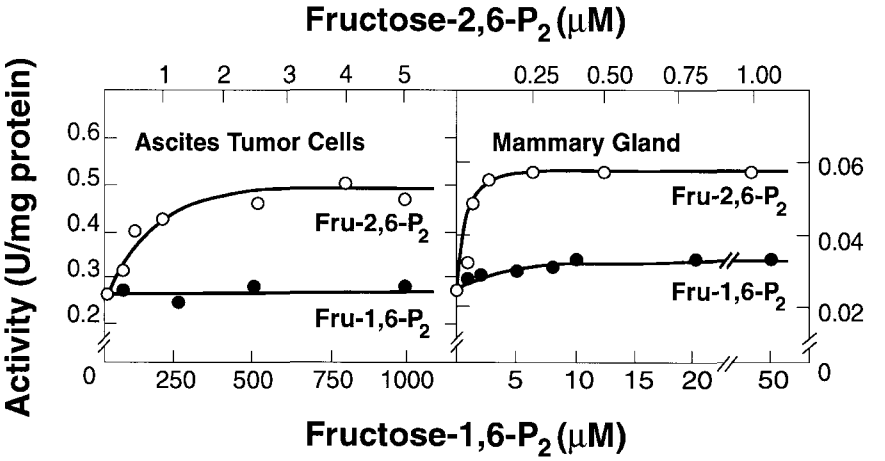

Fig. 3. Effect of fructose-2,6-P_2 and fructose-1,6-P_2 on PFK from murine mammary gland and ascites tumor cells.

3.2. *Effect of fructose bisphosphates and P-enolpyruvate on PFK isozymes*

Ascites tumor PFK was reported [12] to be insensitive to activation by fructose-1,6-P_2, whereas greatly activated by fructose-2,6-P_2, and inhibited by P-enolpyruvate to a higher extent than other isozymes. The finding that PFK from murine mammary gland also contains the M*-type subunit (Fig. 1), led us to investigate whether this PFK exhibits such peculiar regulatory properties in comparison with the ascites tumor isozyme. As shown in Fig. 3, PFK activity in mammary gland was strongly stimulated by fructose-2,6-P_2 (K_{act} = 0.05 μM), but it was also scarcely activated by fructose-1,6-P_2 when assayed under similar conditions. Although it is difficult to extrapolate to the kinetic behavior of the individual subunits because of the possible influence of subunit–subunit interactions in the hybrid forms, these results suggest that the C-type subunits do not contribute to fructose-1,6-P_2 activation, as this was the predominant subunit type in ascites tumor cells (Fig. 1). In fact, C_4 homotetramers (or F_4 homotetramers, according to the nomenclature of Khan et al. [3]) were shown by Meienhofer et al. [6] to be insensitive to activation by glucose-1,6-P_2, which apparently acts similarly to fructose-1,6-P_2 although with lower affinity [25,26]. Muscle PFK (M_4 homotetramers) shows the greatest affinity for fructose-1,6-P_2 among PFK isozymes [26,27]. The liver enzyme is also activated by fructose-1,6-P_2, but only under certain conditions and with a poorer affinity [25]. The fact that mammary gland PFK was only slightly activated by fructose-1,6-P_2, despite containing as much as a 65% of L-type subunits (Fig. 1), may indicate that this subunit type is very little affected by fructose-1,6-P_2, which main target for PFK activation would therefore be the M-type subunit. In addition, the practical lack of fructose-1,6-P_2 effect on mammary gland PFK also suggests that the M*-type subunit is unaffected by this compound, since this isozyme has an amount of the M*-type subunit (33%) even higher than that of ascites tumor PFK (19%), whereas the brain isozyme, which contains a 49% of the normal M-type subunit [1], was found to be highly activated by fructose-1,6-P_2 under similar conditions (data not shown). Consequently, the insensitivity of ascites tumor PFK to fructose-1,6-P_2 activation might just reflect its subunit composition, i.e. a high proportion of the C-subunit type, a relatively lower content of the L-type subunit and the presence of the variant M*-type subunit.

Fig. 4 shows that mammary gland was also inhibited by P-enolpyruvate, exhibiting an affinity for this compound (K_i = 0.83 mM) that practically coincided with that of the ascites tumor isozyme. Thus, these data suggest that both the presence of the M*-type subunit detected in ascites tumor cells and the peculiar kinetic properties initially reported [12] for its PFK activity about fructose-1,6-P_2 activation and P-enolpyruvate inhibition, are not related to some malignant phenotype of these cells but to the tissue from where the tumor proceeds [17,18], whereas the high content of the C-type subunit is apparently linked to the tumorous character of this type of cell. There has been previous studies [3,13–16] on the nature of PFK isozymes in cancer cells, and fructose-2,6-P_2 activation of the rat mammary gland isozyme was described [28], but to the best of our knowledge this is the first determination of the subunit composition of PFK in tumor cells and mammary gland and of the effect of fructose-1,6-P_2 and P-enolpyruvate on the enzyme from the latter tissue.

3.3. *Conclusions*

The results presented here show that PFK isozymes in as-

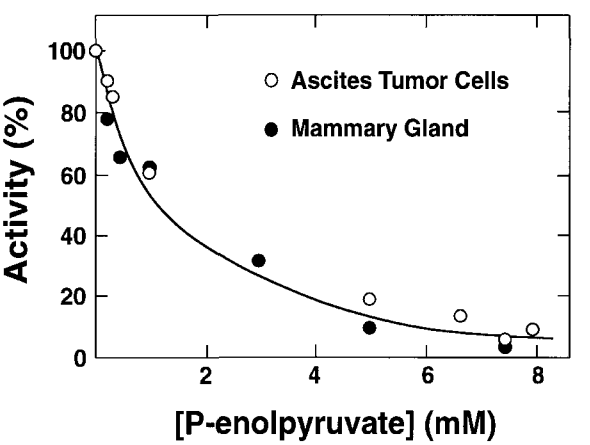

Fig. 4. Effect of P-enolpyruvate on PFK from murine mammary gland and ascites tumor cells. 100% activity is represented by the highest velocity measured with each enzyme.

cites tumor cells contain mainly C- and L-type subunits and to a lesser extent a variant of the M-type subunit of lower electrophoretic mobility, referred to as M*-type subunit, which was also found in murine mammary gland, the original tissue of this tumor [17,18]. The L-type subunit was the more abundant subunit type in mammary gland. No M_4 homotetramers were detected either in ascites tumor or mammary gland by column chromatography, this isozyme form being also apparently absent in brain and liver. As previously described for ascites tumor PFK [12], the mammary gland isozyme was practically insensitive to fructose-1,6-P_2 activation and equally inhibited by P-enolpyruvate. Our data suggest that the altered M*-type subunit is unaffected by fructose-1,6-P_2 and that of the three distinct subunit types of mammalian PFK, the M-type subunit is mainly responsible for activation by this compound. Additionally, these results indicate that the high proportion of the C-type subunit found in ascites tumor might be an expression of its malignant nature, whereas the presence of the M*-type subunit and the peculiar response to fructose bisphosphates and P-enolpyruvate of the PFK activity in these cells are related to the tissue from where the tumor derived.

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