High Expression of Inducible 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase (iPFK-2; PFKFB3) in Human Cancers

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High Expression of Inducible 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase (iPFK-2; PFKFB3) in Human Cancers[1]

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ABSTRACT

Tumor cells maintain an especially high glycolytic rate to supply the anabolic precursors essential for *de novo* nucleotide synthesis. We recently cloned an inducible isozyme of 6-phosphofructo-2 kinase (iPFK-2) that bears an oncogene-like regulatory element in its mRNA and functions to produce fructose-2,6-bisphosphate, which is a powerful allosteric activator of glycolysis. Rapidly proliferating cancer cells constitutively express iPFK-2 *in vitro*, and inhibition of iPFK-2 expression decreases tumor growth in experimental animal models. We report herein that the expression of iPFK-2 mRNA and protein, as assessed by *in situ* hybridization and immunohistochemistry, is increased in many human cancers when compared with corresponding normal tissues. In particular, iPFK-2 expression was found to be markedly elevated in multiple aggressive primary neoplasms, including colon, breast, ovarian, and thyroid carcinomas. iPFK-2 mRNA and protein expression were induced by hypoxia in cultured human colon adenocarcinoma cells, and an examination of normal lung fibroblasts showed that iPFK-2 and fructose-2,6-bisphosphate levels increased specifically during the S phase of the cell cycle. These data indicate that iPFK-2 is abundantly expressed in human tumors *in situ* and may serve as an essential regulator of glycolysis during cell cycle progression and growth in an hypoxic microenvironment.

INTRODUCTION

Cancer cells maintain an abnormally high glycolytic rate even in the presence of oxygen, a phenomenon first described by Otto Warburg over 75 years ago and known as the Warburg effect (1). High glycolytic flux is essential for tumor growth and *in situ* glucose uptake, and lactic acid levels accurately predict tumor progression, invasiveness, metastatic tendency, and overall patient mortality and morbidity (2–7). Glycolytic flux is primarily controlled by the allosteric inhibitory effects of ATP on PFK-1[4], the rate-limiting step of glycolysis (8, 9). F2,6BP is a potent allosteric activator of PFK-1, and it can override the inhibitory effects of ATP on PFK-1 (10). F2,6BP production is increased in several transformed cell lines (11–14) and is induced by oncogenic transformation (*e.g.*, by v-*src*, v-*fps*, and v-*ras*; Refs. 15, 16). Steady-state levels of F2,6BP are dependent on the activity of the bifunctional enzyme PFK-2 (17), but the particular PFK-2 isozyme usurped by cells during neoplastic transformation has remained elusive until recently.

As a result of a genomic search for early response genes, we recently cloned a novel PFK-2 isoform, termed iPFK-2, which is distinguished by the presence of multiple copies of the AUUUA sequence in the 3′UTR of its mRNA (18). The AUUUA motif confers instability and enhanced translational activity to mRNAs and typifies the 3′UTR structure of several proto-oncogenes and proinflammatory cytokines (19). Accordingly, the discovery of AUUUA repeat elements in the regulatory region of a gene for a glycolytic enzyme was notable. The iPFK-2 mRNA transcript is encoded by a single gene termed *PFKFB3* [also referred to as ubiquitous PFK-2 (20), placental PFK-2 (21), and PRG1 (22)], that is localized on chromosome 10p15-p14 (20). Three additional PFK-2 isozymes (PFKFB1, PFKFB2, and PFKFB4) with distinct activities and tissue expression profiles also have been identified (23–25). Of the four PFK-2 isozymes, only PFKFB3 lacks a critical serine phosphorylation site that is required for the down-regulation of kinase activity (26). Accordingly, PFKFB3 has the highest kinase/phosphatase activity ratio of all of the PFK-2 isoforms discovered to date, which is consistent with its role as a powerful activator of glycolysis (26).

iPFK-2 (PFKFB3) mRNA and protein expression and intracellular F2,6BP levels are undetectable in quiescent peripheral blood monocytes but increase appreciably upon proinflammatory activation (18), suggesting that this gene is activated in a manner analogous to that of other early response genes. By contrast, iPFK-2 mRNA and protein expression are constitutively expressed in several transformed cell lines when compared with nontransformed cells (18). Moreover, antisense iPFK-2 oligonucleotide transfection of K562 leukemia cells causes a marked inhibition of cell proliferation and a decrease in steady-state levels of 5-phosphoribosyl-1-PP_I, a glucose-derived precursor that is required for the committed first step of *de novo* purine and pyrimidine synthesis (18). Lastly, antisense iPFK-2 treatment *in vivo* significantly suppresses the outgrowth of human K562 tumors implanted in nude mice, thereby supporting the critical role of this regulatory enzyme in tumor cell metabolism *in vivo* (18).

In this report, we have examined 60 primary human solid tumors and corresponding normal tissues and found that iPFK-2 mRNA and protein is expressed at especially high levels by neoplastic cells *in situ*. Additionally, we show that iPFK-2 expression is up-regulated in response to hypoxic challenge and during the S phase or DNA synthesis phase of the cell cycle.

MATERIALS AND METHODS

Tissue Samples. Formalin-fixed, paraffin embedded normal human and tumor tissue was obtained from Novagen (Madison, WI), DAKO (Carpinteria, CA) and GENPAK (St. James, NY).

***In Situ* Hybridization.** The antisense and sense RNA probes for iPFK-2 were 1.6 kb in length and designed to contain the AU-rich motif in the 3′UTR (corresponding to nucleotides 2557-4162; GenBank accession no. AF056320). The probes were synthesized with T7 polymerase using ^{35}S-CTP and alkali hydrolyzed before use so as to generate probes of ~200–300 nucleotides in length. Tissue sections were deparaffinized with xylene and then pretreated with proteinase K at 37°C for 15 min. The sections then were incubated with 0.1 M triethanolamine buffer and acetylated with 0.12% acetic anhydride in 0.1 M triethanolamine buffer to reduce the nonspecific binding of the probe. Prehybridization was performed with hybridization solution containing 0.3 M NaCl, 0.5 mM EDTA (pH 8.0), 10 mM Tris-Cl (pH 7.4), 0.1% BSA, 0.02%

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[4] The abbreviations used are: PFK-1, 6-phosphofructo-1-kinase; F2,6BP, fructose-2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; iPFK-2, inducible PFK-2; 3′UTR, 3′ untranslated region; FBS, fetal bovine serum; F6P, fructose 6-phosphate; HIF-1, hypoxia inducible factor 1.

Ficoll, 0.2% polyvinylpyrolidone, 5 mM DTT, 50% deionized formamide, and 50 μg/ml mRNA for 2 h at 45°C. Hybridization was performed with ^{35}S-labeled sense or antisense RNA probes at 1.6×10^5 cpm/μl in hybridization solution containing 10% dextran sulfate for 16 h at 45°C. After hybridization, the sections were washed in 2× SSC/1 mM EDTA/5 mM DTT for 15 min at room temperature and then in 50% formamide/1× SSC/0.5 mM EDTA for 15 min at 45°C. The slides were washed three times in 2× SSC/1 mM EDTA/0.1% Triton X-100/5 mM DTT for 15 min at 60°C and twice in 0.1× SSC/1 mM EDTA/5 mM DTT for 15 min at 60°C. The slides then were incubated for 40 min in 25 μg/ml RNase A and 0.25 unit/μl RNase T1 at 37°C. Finally, the slides were washed twice in 2× SSC/1 mM EDTA/5 mM DTT at 60°C, dehydrated, dipped in NTB-3 emulsion autoradiography (Eastman Kodak, Rochester, NY), allowed to dry, and exposed in the dark at 4°C for 3–10 days. The emulsion was developed with D19 developer (Eastman Kodak) and counterstained with H&E and observed under the microscope.

Immunohistochemistry. Five-μm sections were treated with xylene to remove paraffin, rehydrated, and treated with 0.3% hydrogen peroxide for 30 min to eliminate endogenous peroxidase activity. The sections then were blocked by incubation with 1.5% normal goat serum for 20 min at room temperature. After washing, the sections were treated with a rabbit polyclonal anti-iPFK-2 antibody raised to the recombinant protein (1:200 dilution for 30 min). Immunoblotting of human tissue extract (brain, kidney, and liver) and recombinant iPFK-2 protein demonstrated that anti-iPFK-2 antibody recognized a single species with no cross-reactivity (Ref. 18; data not shown).

The tissue sections were treated with Vectastain Elite ABC kit (Vector Laboratories) according to manufacture's recommendation. The negative control included substitution of the primary antibody with nonimmune serum and incubation with anti-iPFK-2 antibody in the presence of excess recombinant iPFK-2. The sections were subsequently incubated with biotinylated, goat antirabbit immunoglobulin (Vector Laboratories, Burlingame, CA) and developed with an avidin-biotin peroxidase reaction using 3,3′-diaminobenzidine tetrahydrochloride as chromogen. After counterstaining with Mayer's hematoxylin (Sigma, St. Louis, MO), the sections were dehydrated, and a coverslip was attached with Permount (Fisher Scientific, Pittsburgh, PA). The intensity of the immunoreactions were graded in a blinded fashion as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3).

Cell Culture. Human lung fibroblasts (Hs 218.Lu) and the colon adenocarcinoma cell line (SW 620) were obtained from the American Type Culture Collection (Manassas, VA). The SW 620 cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT) at 37°C in a humidified 5% CO_2 incubator. Cells (2×10^5/ml) were subjected to hypoxia using the GasPak Pouch (Becton Dickinson, Sparks, MD) according to manufacture's protocol for the times indicated in the figures.

For cell cycle analysis, Hs 218.Lu cells were treated as described previously (16). In brief, 2×10^5/ml cells were cultured in DMEM/0.5% FBS for 48 h to induce growth arrest (G_0-G_1 phase). The cells then were incubated in DMEM/10% FBS for 6 h (G_1 phase) or for 16 h in the presence of 5 μg/ml aphidicoline (G_1-S phase; Calbiochem, San Diego, CA). The aphidicoline then was washed away, and the cells were reincubated for 5 h in DMEM/10% FBS (S phase) or for 20 h in DMEM/10% FBS containing 200 ng/ml colchicine (Calbiochem). The incorporation of [^{3}H]thymidine (4 μCi/ml) into DNA was measured during the last 4 h of incubation at each stage of the cell cycle.

Intracellular F2,6BP levels were measured using Van Schaftingen's method after the disruption of 1×10^6 cells in 0.8 ml of 50 mM NaOH (17).

Northern Blotting. The human iPFK-2 cDNA containing the 3′UTR AU-rich domain (corresponding to nucleotides 2557–4162, as described above) was cloned into pCRII (Invitrogen, Carlsbad, CA), and antisense RNA probes were synthesized with the DIG RNA Labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Total RNA was extracted with an RNeasy Mini kit (Qiagen, Chatsworth, CA). Equal amounts of total RNA (10 μg/lane) were electrophoresed and transferred onto nylon membrane using NorthernMax kit (Ambion, Austin, TX). The membrane was UV cross-linked. Prehybridization was carried out in a hybridization solution (DIG easy Hyb; Roche, Indianapolis, IN) at 68°C for 4 h. The blot was hybridized overnight with DIG-labeled RNA probes at 68°C and washed twice in 2× SSC containing 0.1% SDS at room temperature for 15 min, then washed twice in 0.1× SSC containing 0.1% SDS at 68°C for 15 min. Detection of DIG-labeled RNA probes were carried out using DIG Wash and Block Buffer Set, alkaline phosphatase-conjugated antidigoxigenin antibody and CSPD (Roche Molecular Biochemicals) according to manufacture's protocol.

Western Blotting. Cells were washed in cold PBS and then radioimmunoprecipitation assay buffer containing protease inhibitor (Complete, Mini, EDTA-free; Roche Molecular Biochemicals) was added. The cells were disrupted by repeated aspiration through a 21-gauge needle. After incubation on ice for 30 min, the protein concentration was determined and the samples were mixed with an equal volume of 2× Laemmli loading buffer. The samples were denatured for 5 min, and proteins were separated by 10% SDS-acrylamide electrophoresis gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated with a polyclonal anti-iPFK-2 antibody (1:1000 dilution) that was raised against recombinant human iPFK-2 protein as described above. Bound antibody was visualized with horseradish peroxidase-conjugated donkey antirabbit antibody and enhanced chemiluminescence using the ECL system (Amersham, Buckinghamshire, United Kingdom).

RESULTS

Detection of iPFK-2 mRNA in Primary Human Tumor Tissues. Our previous studies demonstrated that several human transformed cell lines constitutively express iPFK-2 mRNA *in vitro* (18). We designed a RNA probe that was complementary to the AU-rich element in the 3′UTR of iPFK-2 and developed an *in situ* hybridization method to detect iPFK-2 mRNA expression *in situ*. Our initial studies in a selection of different tumor biopsies ($n = 8$) revealed iPFK-2 mRNA to be uniformly increased in the malignant tissues when compared with corresponding control tissues (data not shown). Fig. 1 shows representative *in situ* hybridizations for a prostate adenocarcinoma and a gall bladder carcinoma. iPFK-2 mRNA is readily

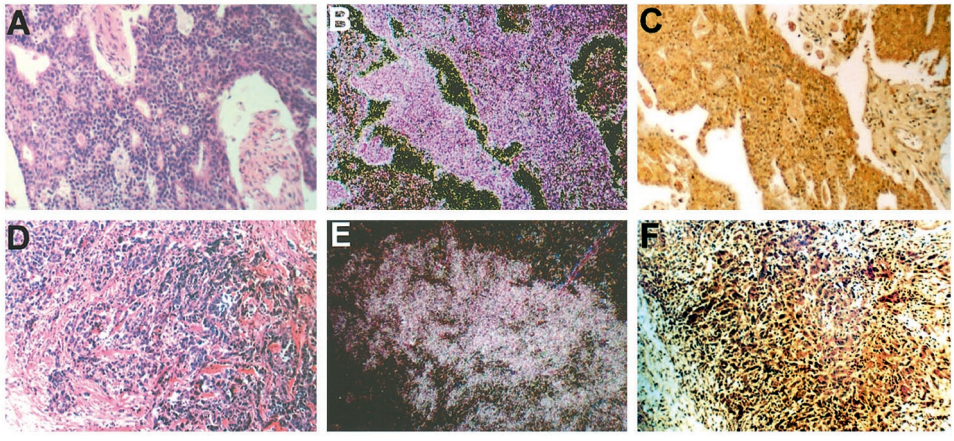


Fig. 1. Analysis of iPFK-2 expression in prostate adenocarcinoma (*A–C*) and gall bladder carcinoma (*D–F*). Tissue sections were examined by H&E (*A* and *D*), *in situ* hybridization (*B* and *E*), and immunohistochemistry using a polyclonal anti-iPFK-2 antibody raised to recombinant iPFK-2 (*C* and *F*). Magnification ×100.

detectable within the neoplastic cells of these solid tumors, and no cell-associated signals were detected with the iPFK-2 sense probe (data not shown). Moreover, we found strong iPFK-2 protein expression *in situ* by the neoplastic cells of these same solid tumors (Fig. 1, *C* and *F*). No detectable immunoreactivity was observed in sections incubated with nonimmune serum instead of primary antibody or in sections incubated with anti-iPFK-2 antibody in the presence of excess recombinant iPFK-2 (data not shown). The pattern of iPFK-2 mRNA and protein expression was similar, further validating the specificity of the anti-iPFK-2 antibody.

iPFK-2 Protein Expression in Normal and Tumor Tissues. On the basis of our preliminary findings that iPFK-2 is expressed by certain tumors *in situ*, we conducted a more comprehensive survey of iPFK-2 protein expression in solid tumors and matched, normal tissue counterparts. We analyzed 60 individual human tumor specimens that were obtained from 16 histologically distinct tumor types and quantified the intensity of staining. A high level of cytoplasmic staining was observed in the majority of the specimens, and the ubiquitous distribution of staining contrasted sharply with the largely focal and epithelial pattern of iPFK-2 immunoreactivity present in normal tissues (Fig. 2; *arrows* indicate epithelial cells). Overexpression of iPFK-2 protein *in situ* was especially high in cancers of the colon, prostate, breast, ovary, and thyroid (Figs. 2 and 3). Sections from colon adenocarcinoma, for instance, showed relatively strong immunoreactivity for iPFK-2 when compared with the adjacent, morphologically normal mucosa in the same specimen (Fig. 3*A*). In breast carcinoma, aggressive infiltrating carcinoma cells were found to be markedly positive for iPFK-2 protein and adjacent normal epithelium within the same specimen displayed only weak staining (Fig. 3, *B–D*). We compared the relative expression of iPFK-2 protein between neoplastic cells and matched, iPFK-2-positive normal cells and found that within a particular tumor type, neoplastic cells express higher iPFK-2 than adjacent epithelial cells (Fig. 4). Taken together, these data indicate that neoplastic cells constitutively express increased iPFK-2 protein *in situ* when compared with cells from matched, normal tissues.

Effect of Hypoxia on iPFK-2 Expression *in Vitro*. The high growth rate of certain solid tumors can be restricted by the host angiogenic response, leading to a hypoxic environment that obliges transformed cells to rely on glycolysis for ATP production (27). Given the unique ability of the product of iPFK-2 F2,6BP to activate glycolytic flux, we hypothesized that a hypoxic environment may induce iPFK-2 expression.

We investigated iPFK-2 mRNA and protein expression in SW 620 human colon adenocarcinoma cells after culture in ambient oxygen or under conditions of hypoxia. Hypoxia induced a significant and time-dependent increase in cellular iPFK-2 mRNA over the 14-h study period (Fig. 5*A*). This effect was accompanied by a concomitant increase in intracellular iPFK-2 protein levels (Fig. 5*B*). Surprisingly, despite the increased expression of iPFK-2 protein in these cells, the intracellular content of F2,6BP decreased (Fig. 5*C*). These data suggest that the substrate for iPFK-2, F6P, may be restricted by either decreased production by phosphoglucose isomerase or by increased 1-phosphorylation by PFK-1.

iPFK-2 Expression during Cell Cycle Progression. Enhanced glycolysis is a common feature of proliferating cells and likely functions to provide carbohydrate intermediates for *de novo* nucleic acid synthesis. We postulated that iPFK-2 expression may be up-regulated during the S phase of the cell cycle to increase F2,6BP and drive glycolytic flux. We synchronized normal human lung fibroblasts (Hs 218.Lu cell line) in culture, verified their growth phase by [^{3}H]thymidine incorporation, and measured iPFK-2 mRNA and protein levels and intracellular F2,6BP levels. As shown in Fig. 6, iPFK-2 mRNA expression was induced during the G_1-S and S phases, and iPFK-2 protein expression was highest during S phase. Intracellular F2,6BP levels paralleled iPFK-2 protein expression and thus were increased during the S phase of the cell cycle (Fig. 6*C*). Taken together, these data suggest that iPFK-2 activity is tightly controlled during the cell cycle and that high protein expression of this enzyme occurs concurrently with DNA synthesis.

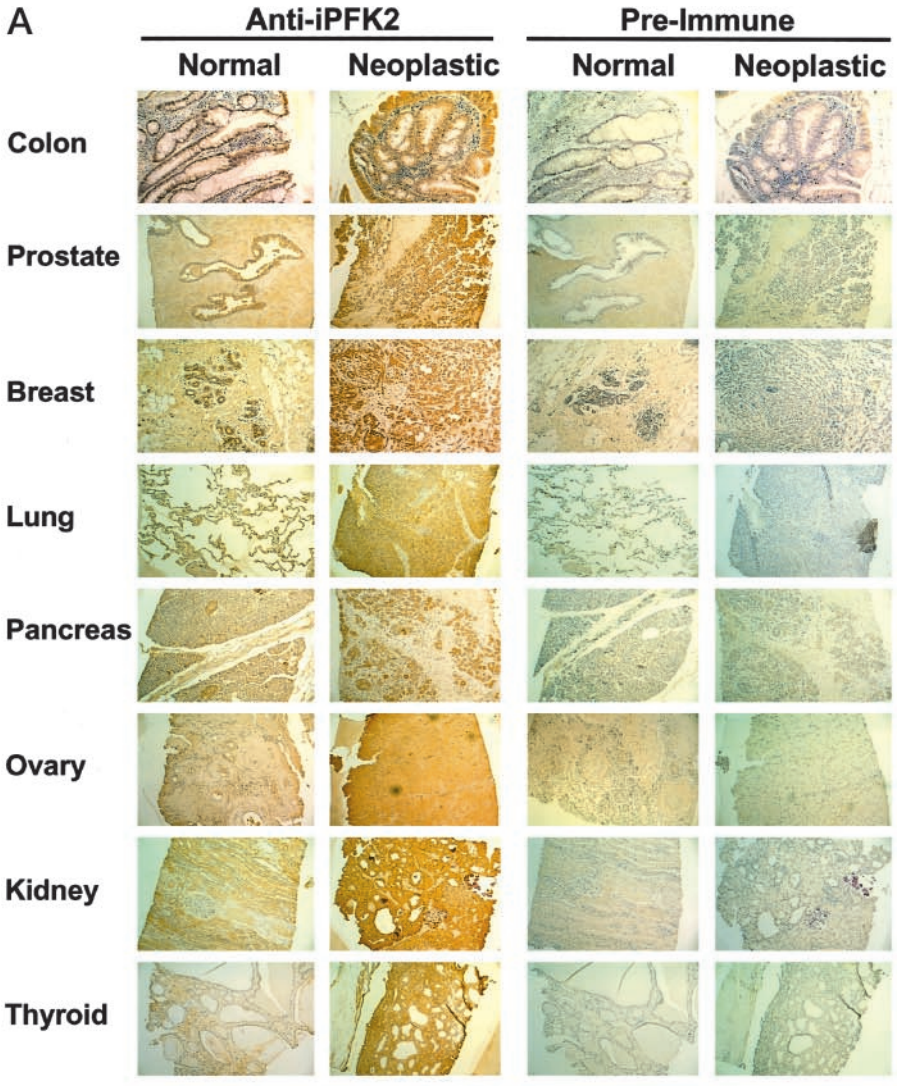
DISCUSSION

In the late 19th century, Louis Pasteur demonstrated that the consumption of carbohydrates by yeast is decreased 7-fold in the presence of oxygen (28). This phenomenon became known as the Pasteur effect, and it is considered essential for coupling the rate of glucose breakdown (to pyruvate) with acetyl-CoA entry into the tricarboxylic acid cycle during respiration. Under adequate oxygen supply, ATP is generated in abundance by the respiratory chain, and ATP, by an allosteric interaction, directly inhibits PFK-1, which is the rate-limiting enzyme in glycolysis (9). In the 1920s, Otto Warburg discovered that human tumors exhibit an abnormally high rate of glucose catabolism in the presence of oxygen (*i.e.*, aerobic glycolysis; Ref. 1). Warburg believed that cancer cells suffer from mutations that cause altered respiratory function and thus are obligated to derive energy exclusively from glycolysis.

Positron emission tomography with 2-[^{18}F]fluoro-2-deoxy-D-glucose has demonstrated that human tumors uniformly metabolize about 10-fold more glucose than normal tissues *in situ*, regardless of their cell type or organ (2, 3, 6, 29). Moreover, the rate of glucose metabolism directly correlates with tumor aggressiveness (*i.e.*, growth rates, invasiveness, and metastatic potential) and with overall patient morbidity and mortality (4, 5). This metabolic disturbance is particularly surprising because satisfying the energy demands of the cell with glycolysis alone is an inefficient process and produces excess H^+ ions, thereby decreasing the extracellular pH and threatening cellular integrity (30, 31). Although the precise reasons for increased aerobic glycolysis by cancer cells are unknown, the main products of glycolysis, ATP and carbohydrate precursors for the synthesis of nucleic acids and amino acids, are essential for rapid cell proliferation (28).

Until recently, the specific regulatory mechanisms responsible for increased glycolysis in the presence of oxygen have remained largely obscure. Increased cell surface expression of glucose transporter 1 (7, 32) and type II hexokinase activity (33–35) in cancer cells has been found to be necessary for high glycolytic flux. High glucose transporter 1 expression enables cancer cells to be freely permeable to extracellular glucose, and the activities of downstream glycolytic enzymes thus control the rate of glucose flux. Hexokinase phosphorylates glucose to form glucose 6-phosphate, which can undergo three possible fates: conversion into glycogen; oxidation by the pentose phosphate pathway to generate NADPH; or, as observed in rapidly proliferating cancer cells, isomerization to F6P. Although hexokinase is the first irreversible step of glycolysis, it is not the rate-limiting step because of the several fates of its product. Rather, PFK-1 is the first irreversible and committed step of glycolysis, and this enzyme thus dictates the rate of glycoytic flux (9, 17, 28). PFK-1 activity is modulated by several allosteric effectors, including ATP (*i.e.*, the Pasteur effect), H^+ ions, and citrate, which creates negative feedback when energy is abundant (9, 17, 28). Importantly, PFK-1 activities are markedly increased in both cancer cell lines and primary tumor tissues *in situ* (36–38).

In 1980, a novel allosteric regulator of PFK-1 and glycolysis was discovered by Van Schaftingen *et al.*: F2,6BP (10). F2,6BP allosterically activates glycolysis by shifting the conformational equilibrium of PFK-1 from a low to a high affinity state for its substrate, F6P (10,



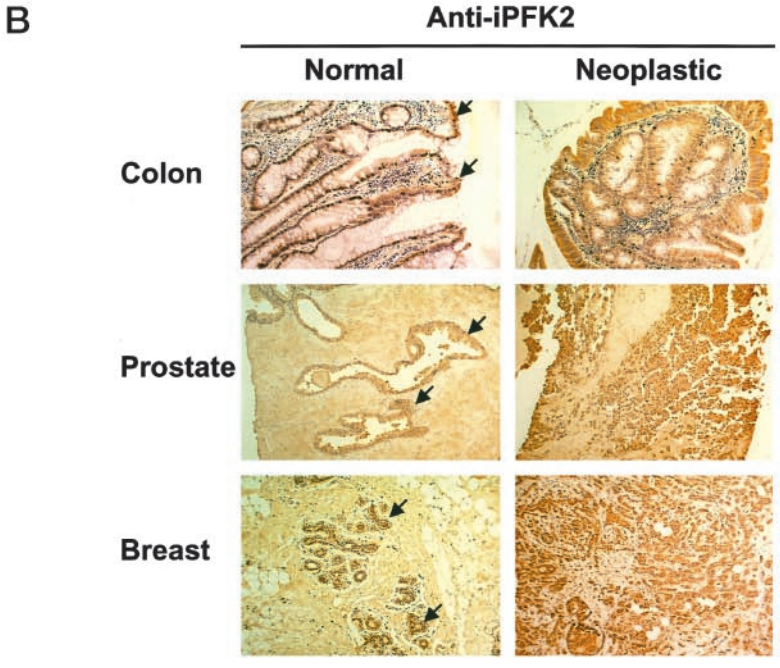


Fig. 2. Immunohistochemical analysis of iPFK-2 protein expression by common human cancers and their normal tissue counterparts. Tissue sections of neoplastic and matched, normal tissues were examined by immunohistochemistry using either an anti-iPFK-2 polyclonal antibody or preimmune rabbit serum. *A*, magnification ×40; *B*, magnification ×100 (*arrows* indicate epithelial cells).

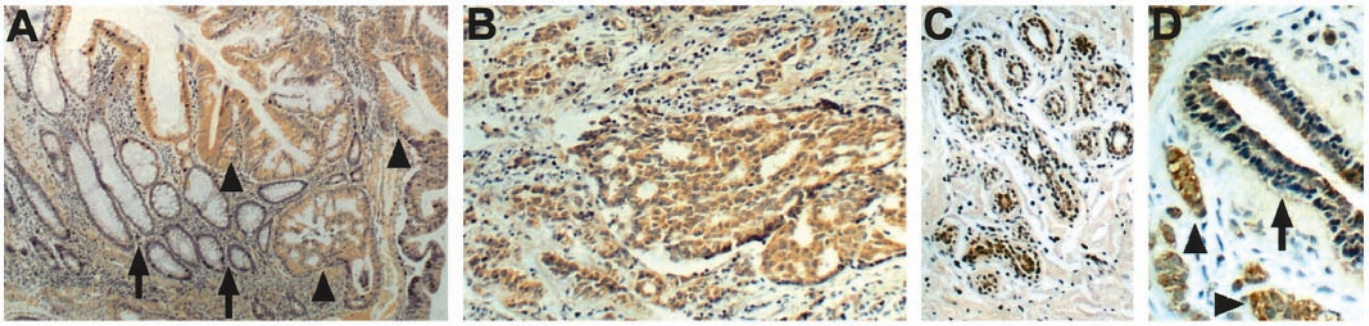


Fig. 3. Direct comparison of iPFK-2 protein expression between normal and cancerous tissues within the same tissue section. *A*, colon normal tissue and adenocarcinoma. *B*, breast adenocarcinoma. *C*, adjacent normal breast epithelium in the same specimen displayed in *B*. *D*, breast normal tissue and adenocarcinoma. Magnification ×40 (*A* and *C*) and ×100 (*B* and *D*).

17). Micromolar intracellular concentrations of F2,6BP can relieve the tonic allosteric inhibition by ATP on PFK-1 that occurs in the presence of oxygen (10, 17). The steady-state concentration of F2,6BP depends on the activity of the homodimeric bifunctional enzyme PFK-2), which is expressed in several tissue-specific isoforms (23–25). Of importance, multiple established cancer cell lines (*i.e.*, Ehrlich ascites tumor cells, HeLa cells, HT29 colon adenocarcinoma cells, Lewis lung carcinoma cells, HL60 cells, SW480 colon adenocarci-

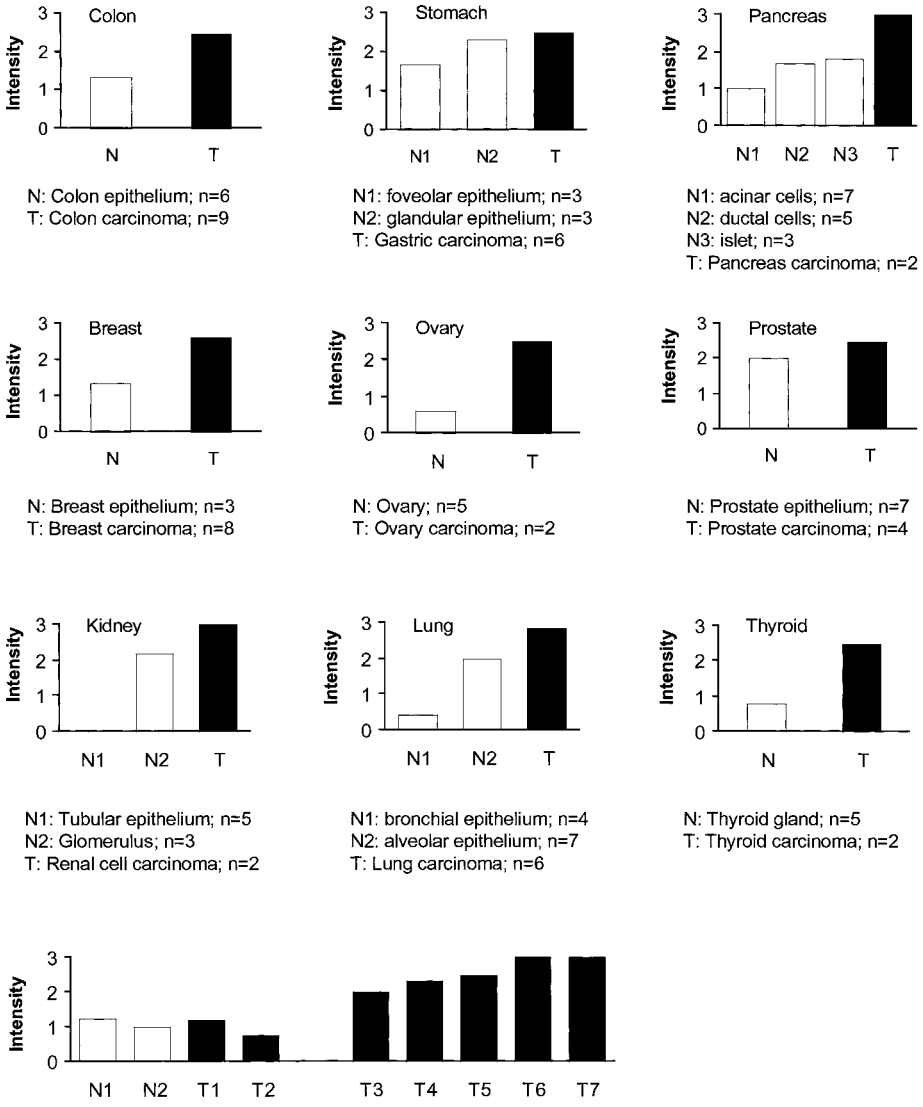


Fig. 4. Mean expression score of iPFK-2 protein by cells within 132 specimens of human normal and cancerous tissues. The intensity of the immunoreactions were graded as described in "Materials and Methods" as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3).

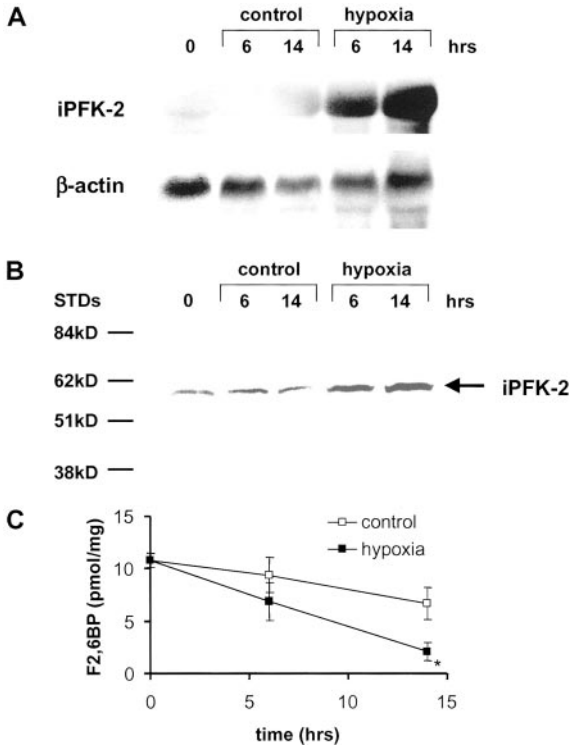


Fig. 5. Effect of hypoxia on the expression of iPFK-2 in SW 620 colon adenocarcinoma cells. *A*, Northern blot analysis for iPFK-2 mRNA. *B*, Western blot analysis for iPFK-2. *C*, intracellular [F2,6BP]. Values represent mean ± SD of three independent experiments. ∗, $P < 0.05$.

noma cells, A549 lung adenocarcinoma cells, and K562 leukemia cells) have markedly elevated levels of F2,6BP when compared with their normal tissue counterparts (11–14, 18). Furthermore, transformation of chick embryo fibroblasts by retroviruses carrying either the v-*src* or v-*fps* oncogenes induces F2,6BP synthesis and causes increased glycolytic flux and cell proliferation (15). Whereas the allosteric actions of F2,6BP had been implicated in the observed high glycolytic flux of neoplastic cells, the particular PFK-2 isozyme responsible for malignant F2,6BP production has only recently been identified.

Rapidly proliferating transformed cells constitutively express iPFK-2 (PFKFB3) mRNA and protein *in vitro*, and inhibition of iPFK-2 expression decreases tumor growth in experimental animal models (18). We now find that iPFK-2 mRNA and protein are expressed at high levels *in situ* by the neoplastic cells of several primary human solid tumors. The observation that iPFK-2 is constitutively expressed by neoplasms *in situ* and that its product, F2,6BP, functions to activate glycolytic flux reinforces the concept that the high glycolytic flux of neoplastic tissues is regulated via this pathway.

The regulation of iPFK-2 activity *in vivo* is likely the result of the net effect of transcriptional mediators (*e.g.*, DNA and mRNA/AU-binding factors) and posttranslational modifications (*e.g.*, serine kinases). Of note, *ras*-transformation in rat-1 fibroblasts has been demonstrated previously to induce high intracellular F2,6BP levels and aerobic glycolysis (16). Approximately 25% of all human tumors express mutated, activated *ras*, and 50% of human colon carcinomas bear mutant *ras* oncogenes (39). *Ras* activation of the extracellular signal-regulated/mitogen-activated protein kinase cascade leads to increased gene expression in part through the action of the transcription factors *myc* and nuclear factor κB, both of which have multiple potential binding sites on the iPFK-2 promoter (GenBank accession no. AF11058; Refs. 40–42).

Recently, Minchenko *et al.* (43) demonstrated that human hepatoma cells up-regulate iPFK-2 (PFKB3) mRNA in response to hypoxia and that the transcription factor HIF-1 is required for this induction in mouse fibroblasts. HIF-1 target genes are critical for neoplastic growth because disruption of HIF-1-promoting activity suppresses tumor growth *in vivo* (44). We have demonstrated that both iPFK-2 mRNA and protein expression are increased in response to prolonged hypoxia in SW 620 colon adenocarcinoma cells. However, we find that increased iPFK-2 expression under these experimental conditions is associated with decreased intracellular F2,6BP levels. We hypothesize that the substrate of iPFK-2, fructose-6-phosphate, becomes restricted during exposure to hypoxic conditions.

We also demonstrate that iPFK-2 mRNA and protein expression increases during the S phase of the cell cycle, thus supporting the hypothesis that F2,6BP is required for enhanced flux of carbohydrates into *de novo* nucleic acid synthesis. Interestingly, HuR, one of the AU-rich element binding proteins that affects mRNA stability, has been found to localize to the cytoplasm and to regulate cyclin A and cyclin B1 mRNA stability during the G_1-S phases of the cell cycle (45). Given the large AU-rich element in the iPFK-2 mRNA 3′UTR and the observed increased iPFK-2 expression during the G_1-S transition, we postulate that the HuR protein may also effect transcriptional regulation of iPFK-2.

Using Northern blot analysis, we previously found low constitutive expression of iPFK-2 mRNA in normal human tissues (18). We now report that iPFK-2 protein is nearly ubiquitously expressed by epithelial cells, albeit at lower levels than most neoplastic cells in solid tumors. That epithelial cells use this regulatory pathway to enhance glycolysis is not surprising given their high rate of basal glycolysis and proliferation. Many common solid tumors originate from neoplas-

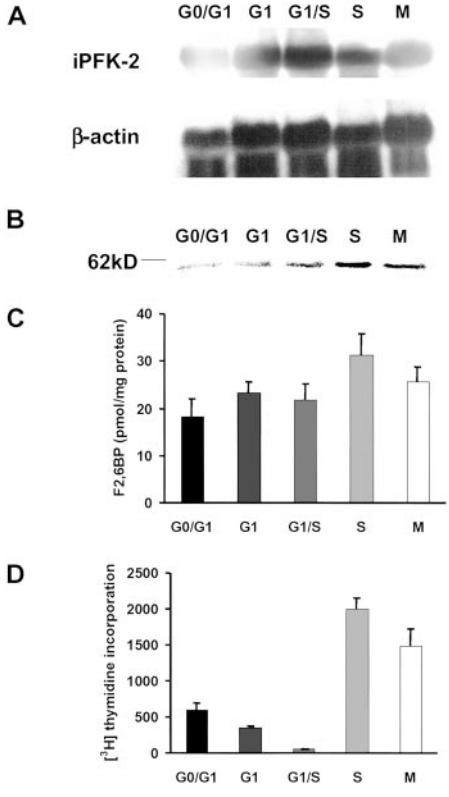


Fig. 6. iPFK-2 expression during cell cycle progression in a synchronized normal human lung fibroblast cell line. *A*, Northern blotting of iPFK mRNA expression. *B*, Western blotting of IPFK-2 protein expression. *C*, intracellular [F2,6BP]. *D*, [^{3}H]thymidine incorporation measurement of synchronized normal human lung fibroblasts. Values represent the mean ± SD of three independent experiments.

tic transformation of epithelial cells (*e.g.*, lung, breast, prostate, and colon adenocarcinomas), and we postulate that transformation capacity may depend, in part, on the metabolic phenotype of the cell before oncogenesis. Accordingly, high baseline iPFK-2 expression may confer a metabolic profile that predisposes epithelial cells to transformation and tumor progression.

In summary, we demonstrate that iPFK-2 is a novel, glyco-regulatory enzyme that is overexpressed by several solid tumors *in situ*, where it appears to function to enhance glycolytic flux and permit rapid cellular proliferation. iPFK-2 may find clinical utility as a novel target for the development of antineoplastic agents.

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