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Phosphorylation of the 6-Phosphofructo-2-Kinase/Fructose 2,6-Bisphosphatase/PFKFB3 Family of Glycolytic Regulators in Human Cancer

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Abstract

Purpose: Fructose 2,6-bisphosphate (F2,6BP) is a potent activator of phosphofructokinase, which is a rate-limiting enzyme of glycolysis. The concentration of F2,6BP depends on the activity of the bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase). Four genes encoding PFK-2/FBPase have been identified and termed *PFKFB1* to *PFKFB4*. PFKFB3 protein is expressed in high levels in human tumors *in situ*. The purpose of this study was to determine the role of functional interactions between the phosphorylation of PFKFB3 and activated glycolysis in human cancer cells.

Experimental Design: cDNA from several human tumor cell lines and human colon carcinoma were analyzed by reverse transcription-PCR to identify different splicing variants of PFKFB3. The effect of phosphorylation of Ser^{461} was studied by recombinantly replacing this residue with glutamate ($PFKFB3^{S461E}$). The phosphorylation of PFKFB3 protein in human cancer was determined by immunostaining using an anti-phospho-PFK-2(PFKFB3) antibody.

Results: Two splicing variants of PFKFB3 are expressed in human cancer cell lines: PFKFB3-ACG and PFKFB3-AG. Quantitative, real-time PCR analysis confirmed the overexpression of PFKFB3 mRNA in colon carcinoma, with the dominant variant being the PFKFB3-ACG isoform that contains a phosphorylation site at Ser^{461}. Forced expression of PFKFB3-ACG in COS-7 cells resulted in enhanced glycolysis. Introduction of $PFKFB3\text{-}ACG^{S461E}$ into COS-7 cells led to increased the lactate production and cell proliferation. Highly phosphorylated PFKFB3 protein was found in human tumor cells, vascular endothelial cells, and smooth muscle cells, as determined by immunostaining with an anti-phospho-PFK-2(PFKFB3) antibody.

Conclusions: These findings support a potential role for the phosphorylation of PFKFB3 protein in the progression of cancer and angiogenesis.

Cancer cells maintain a high glycolytic rate even in the presence of oxygen, a phenomenon first described over 70 years ago and known historically as the Warburg effect (1). Despite many years of investigation, the specific regulatory mechanisms responsible for the Warburg effect have been unclear. The major regulatory step in glycolysis involves phosphofructokinase-1 (PFK-1) activity, which is controlled by the intracellular ratio of ATP to AMP. High levels of ATP inhibit PFK-1 activity. The intracellular allosteric regulator, fructose 2,6-bisphosphate (F2,6BP), in turn, is a potent activator of PFK-1 (2). F2,6BP increases the affinity of PFK-1 for fructose 6-phosphate and diminishes the inhibitory effect of ATP. The discovery of F2,6BP has led to the concept that intracellular F2,6BP levels regulate the glycolytic rate in proliferating cells by coupling hormonal signals with metabolic demand. The steady-state concentration of F2,6BP in turn depends on the activity of the homodimeric, bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase; ref. 3). Multiple established cancer cell lines produce markedly elevated levels of F2,6BP when compared with normal tissue cells (4–6). These data suggest that activation and/or overexpression of PFK-2-catalyzed F2,6BP synthesis may contribute to transformation or cell proliferation.

At least four genes (*PFKFB1*, *PFKFB2*, *PFKFB3*, and *PFKFB4*) have been identified that encode PFK-2/FBPase (7). Distinct properties characterize these isoforms, including the ratio of their kinase/phosphatase activities, their response to protein kinases, and their tissue expression profiles (8–10). For instance, glucagon activates the cyclic AMP–dependent protein kinase (PKA), which phosphorylates Ser^{32} of the liver PFK-2 (encoded by *PFKFB1*), producing an inhibition of its kinase activity (11). In contrast, PFKFB3 protein lacks a critical serine phosphorylation site, corresponding to Ser^{32} in liver PFK-2, which is required for the down-regulation of kinase activity.

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The PFKFB3 protein is expressed in activated lymphoid cells as well as in different tumor cell lines and is localized on chromosome 10p15p14 (12–14). Accordingly, PFKFB3 protein has the highest kinase/phosphatase activity ratio of all the PFK-2/FPBase isoforms discovered to date, which is consistent with its role as a powerful activator of glycolysis (15).

The *PFKFB3* gene is distinguished from other PFKFB family members by the presence of multiple copies of the AUUUA sequence in the 3′ untranslated region. This AUUUA motif confers instability and enhanced translational activity to mRNAs and typifies the 3′ untranslated region structures of several proto-oncogenes and proinflammatory cytokines (16). Antisense PFKFB3 oligonucleotide transfection of K562 leukemia cells results in a marked reduction of F2,6BP levels, decreased cell proliferation, and inhibition of tumor growth *in vivo* (12). The PFKFB3 protein is known to be expressed at especially high levels by neoplastic cells *in situ* (17), and there is evidence that its expression is up-regulated in response to hypoxic challenge and during the S or DNA synthesis phase of cell cycle (17). In these reports, the *PFKFB3* gene product was termed "inducible" PFK-2 (iPFK-2) due to this molecular feature. On the other hand, alternative splicing of the variable, COOH-terminal region of the human *PFKFB3* gene can lead to the expression of six structural isoforms (18). In a previous report, an RNA probe for PFKFB3 was designed using the 3′ untranslated region containing AU-rich element, which is a common feature among the different splicing isoforms of PFKFB3 (12, 17). Accordingly, data obtained with this mRNA probe do not distinguish between the iPFK-2 and other potential splicing variants. The PFKFB3 protein in turn has been alternatively termed iPFK-2 (12), ubiquitous PFK-2 (19), placenta PFK-2 (20), and brain PFK-2 (21). Although an iPFK-2-specific antibody supported the production of this variant in human tumors (12), the protein product that results from the major splicing variants present in proliferating tumor cells have not been investigated. Alternative splicing variants of rat brain PFKFB3 also have been reported, but not all these variants may be present in human brain (18, 22). Human PFKFB3 variants have been termed UBI2K1 to UBI2K6, rat PFKFB3 variants have been called RB2K1 to RB2K8, and mouse PFKFB3 variants have not yet been classified. A simplified classification therefore is necessary for better investigation of the PFKFB3 protein.

The regulatory mechanism for increasing the kinase activity of PFKFB3 protein involves phosphorylation of the Ser^{461} residue contained within the COOH-terminal, splice-sensitive region (23). The contribution of the phosphorylation of the COOH-terminal serine to the kinetic properties of PFK-2/FBPase is well characterized in the cardiac, PFK-2/FBPase isoform (24). The insulin-induced increase in F2,6BP and PFK-2 activity occurs by the phosphorylation of this serine residue and is abolished by wortmannin, which is consistent with the involvement of phosphatidylinositol 3-kinase in this activation cascade (24). The PFKFB3 protein contains a similar serine phosphorylation site, and it has been reported that its phosphorylation similarly up-regulates the kinase activity of PFKFB3 protein (23).

Previous studies have suggested a potentially critical role for PFKFB3 protein in the metabolic regulation of human cancers (12, 17). In the present study, we have analyzed the expression pattern of the different splicing variants of PFKFB3 and studied the phosphorylation of this bifunctional enzyme in human cancer tissue using a specific, anti-phospho-PFK-2(PFKFB3) antibody.

Materials and Methods

Reagents. Formalin-fixed, paraffin-embedded normal and tumor tissue was obtained from Ambion (Austin, TX) and DAKO (Carpinteria, CA). A human colon-matched cDNA pair panel and human skeletal muscle cDNAs were obtained from BD Biosciences Clontech (Palo Alto, CA). Antibodies directed against phospho-AMPK (Thr^{172}) and AMP-activated protein kinase (AMPK) were obtained from Cell Signaling Technology (Beverly, MA). A rabbit polyclonal anti-phospho-PFK-2(PFKFB3) antibody was raised against the phosphorylated peptide RRN(Sp)VTP (corresponding to residues 458-464 of human PFKFB3 protein in which Ser^{461} was phosphorylated). This peptide is homologous to that containing the Ser^{466} phosphorylation site of heart PFK-2 and is a consensus sequence for phosphorylation by AMPK. A rabbit polyclonal anti-PFK-2(PFKFB3) antibody was raised against the peptide SPEPTKKPRINSFEEHVAS, which corresponds to the COOH-terminal, specific sequence of PFKFB3.

Cell culture. HepG2 liver cells, HCT15 colon carcinoma cells, MCF7 breast carcinoma cells, U937 monocytes, Jurkat T cells, and RAW 264.7 monocytes were a gift from Dr. Jodo (Hokkaido University). HepG2, HCT15, MCF7, and RAW 264.7 cells were grown in DMEM (Sigma, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). U937 and Jurkat cells were grown in RPMI 1640 (Sigma) with 10% heat-inactivated fetal bovine serum. Cells were cultured at 37°C in a humidified 5% CO_2 incubator.

Reverse transcription-PCR. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed with Omniscript Reverse Transcriptase (Qiagen) in a 20-μL reaction mixture using an oligo-$d(T)_{12\text{-}18}$ primer. PCR was carried out with the Platinum PCR SuperMix (Invitrogen). For the amplification of the variable region of human PFKFB3 cDNA, 200 nmol/L of the forward primer (5′-ATCTACCTGAACGTGGAGTCCGTCTG-3′) and the reverse primer (5′-TCAGTGTTTCCTGGAGGAGTCAGC-3′) were used. These primers were designed to encompass the COOH-terminal, variable region of PFKFB3. The PCR conditions were initial denaturation for 3 minutes at 95°C, 38 cycles of 30 seconds at 95°C, 30 seconds at 61°C and 1 minute at 72°C, and finally 10 minutes at 72°C. PCR was done in a Perkin-Elmer model 2400 thermal cycler. PCR products were separated by electrophoresis on a 5% polyacrylamide gel. In accordance with previous findings, the length of the resultant PCR product enables us to distinguish between the different PFKFB3 splicing variants (18). Amplified fragments from each PCR reaction were cloned into pCR2.1 vector (Invitrogen) and sequenced using the dye terminator cycle sequence kit (Applied Biosystems, Foster City, CA).

Quantitative real-time PCR. Human colon cancer and corresponding normal tissue cDNAs from five individuals were purchased from BD Biosciences Clontech. Quantitative real-time PCR for PFKFB3 mRNA was done using the ABI 7000 Sequence Detector (Applied Biosystems) and a QuantiTect SYBR green reverse transcription-PCR kit (Qiagen). The following primers were used for specific amplification of human PFKFB3: 5′-CTGGACAGGGAGGGAGATACTA-3′ and 5′-AATGAAGAGCTTTGCCCGTGGTC-3′ (Genbank accession no. NM_004566). The specificity of each PCR product was verified by melting curve analysis and by agarose gel electrophoresis.

Cloning of mouse PFKFB3. Full-length mouse adipocyte cDNA was amplified with specific primers (5′-ATGCCGTTGGAACTGACCCA-3′ and 5′-GTGCTTCTGGGAAGAGTCGGCAC-3′). PCR was carried out with Advantage 2 PCR Enzyme System (BD Biosciences Clontech) according to the manufacturer's protocol. The PCR products were separated by electrophoresis on 2% agarose gels, purified using GENECLEAN (Qbiogene, Inc., Carlsbad, CA), cloned into the

pcDNA3.1/V5-His vector, and then transformed into TOP10 *Escherichia coli* cells. Purified plasmid DNA (Qiagen) was sequenced bidirectionally using the BigDye Terminator Cycle sequencing kit. The sequencing reaction products were analyzed with an ABI Model 373A DNA sequencer (Applied Biosystems). The entire predicted nucleotide sequences of mouse PFKFB3 were aligned with the DNAstar (Madison, WI) MEGALIGN application.

Mutational modification of PFKFB3. Site-directed mutatagenesis of mouse PFKFB3 to create PFKFB3-ACG^{S461E}, replacing Ser^{461} with glutamate, was done using a QuikChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Briefly, 10 ng of pcDNA3.1/V5-His-PFKFB3 were used as a template. PCR was carried out with mutagenic oligonucleotide primers (5′-CCCGCTCATGCGACGCAATGAAGTCACCCCACTAGCC-3′ and 5′-GGCTAGTGGGGTGACTTCATTGCGTCGCATGAGCGGG-3′). PCR conditions were as follows: denaturation at 95°C for 1 minute followed by 18 cycles of denaturation at 95°C for 50 seconds, annealing at 60°C for 50 seconds, and extension at 68°C for 12 minutes. After digestion with *Dpn*I, 2 μL of PCR products were used to transform XL10-Gold competent cells provided with the kit. Appropriate clones were verified by DNA sequencing.

Transfection of COS-7 cells. COS-7 cells were cultured in 6-well plates in DMEM containing 10% fetal bovine serum and transfection was done using the FuGENE6 reagent (Roche Diagnostics Co., Indianapolis, IN) according to the manufacturer's protocol. After 48 hours, the cells were used for experiments. Transfection efficiencies were calculated by determining the number of transfected cells using β-Gal staining Kit (Invitrogen).

Measurement of fructose-2,6-bisphosphate and lactate. Intracellular F2,6BP content were measured using Van Schaftingen's method after the disruption of cells in 0.8 mL of 50 mmol/L NaOH (25). Lactate concentration in supernatant was measured by Lactate Pro (ARKRAY, Inc., Kyoto, Japan).

Cell proliferation assay. After transfection, COS-7 cells were harvested and seeded in 96-well plates. Cell proliferation was measured by Cell Counting kit-8 (Dojindo, Kumamoto, Japan) according to manufacturer's recommendation. In brief, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] is converted to formazan by cellular dehydrogenase. The absorbance of formazan, which is proportional to cell number, was determined at 450 nm.

Western blot analysis. Cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris (pH 7.5)] containing protease inhibitors (Complete, Mini, EDTA-free, Roche Diagnostics). The cells were disrupted by repeated aspiration through a 21-gauge needle. After incubation on ice for 30 minutes, protein concentration was determined and the samples were mixed with an equal volume of 2× Laemmli sample buffer. The samples were denatured for 5 minutes and the proteins separated by electrophoresis in 10% SDS-polyacrylamide electrophoresis gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated with a polyclonal anti-PFK-2(PFKFB3) antibody or anti-phospho-PFK-2(PFKFB3) antibody (1:1,000). Bound antibody was visualized with horseradish peroxidase-conjugated donkey anti-rabbit antibody and chemiluminescence using the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

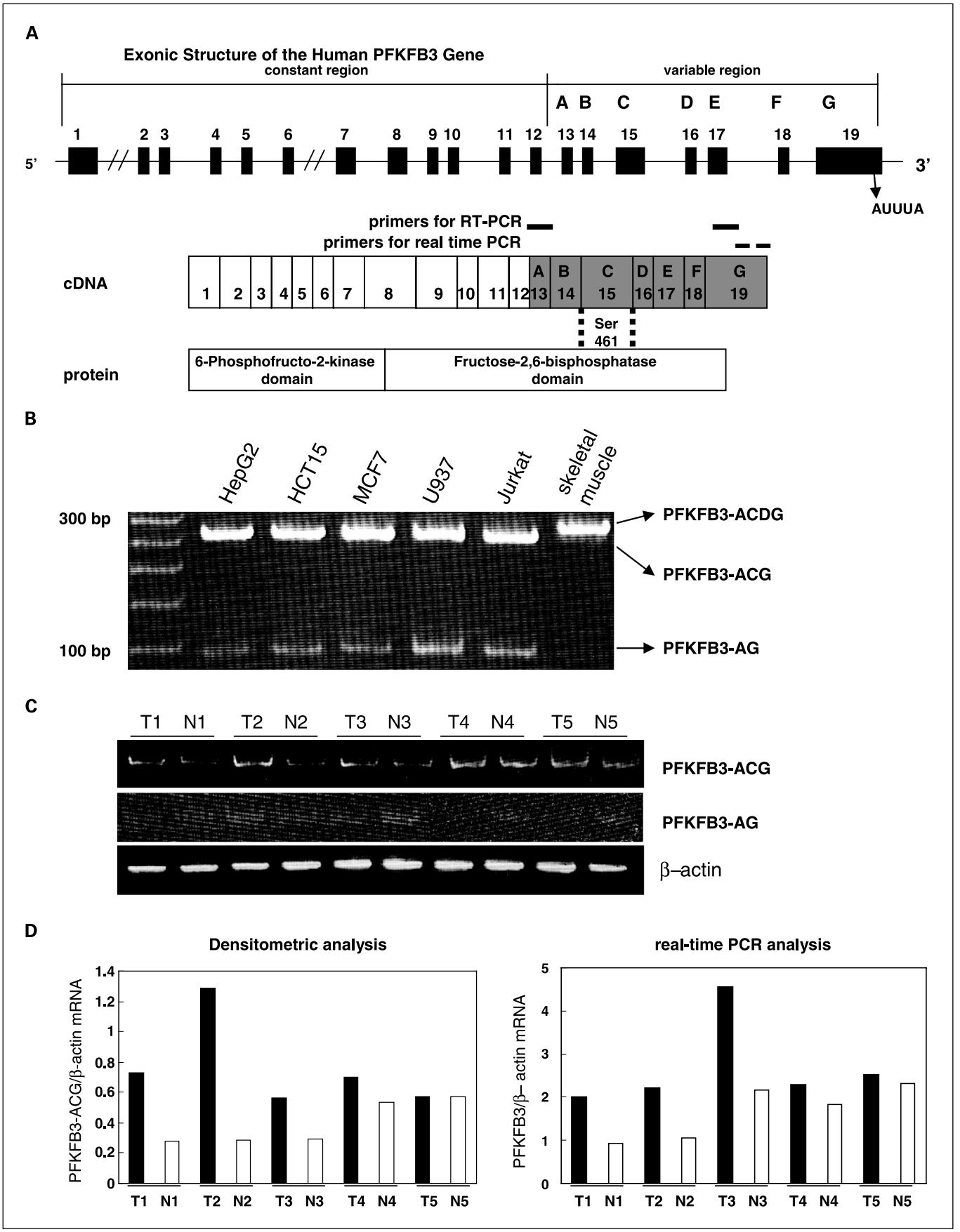
Immunohistochemistry. Immunohistochemistry was carried out as described previously (17). In brief, tissue sections were treated with paraffin, rehydrated, and treated with 0.3% H_2O_2. After blocking with 1.5% goat serum, the sections were treated with rabbit polyclonal anti-phospho-PFK-2(PFKFB3) antibody raised against the phosphorylated peptide (1:200 dilution for 30 minutes). The sections were treated with Vectastain Elite avidin-biotin complex kit (Vector Laboratories, Burlingame, CA) according to manufacturer's recommendation. The intensity of the immunoreactions were graded in a blinded fashion as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3).

Statistical analysis. Results are expressed as means ± SD. Statistical significance of difference was assessed by Student's *t* test and the differences were considered significant if $P < 0.05$.

Results

PFKFB3 mRNA splicing variants are expressed in human tumor cell lines. In a prior study in the human brain, the *PFKFB3* gene has been shown to generate multiple RNA transcripts by alternative splicing (18). The various isoforms are characterized by differences in the length of the COOH-terminal, variable region. The variable region of PFKFB3 is comprised of seven exons (A-G; Fig. 1A). To determine the expression pattern of splicing variants of PFKFB3 in various human tumor cell lines, we did reverse transcription-PCR analysis using primers designed to distinguish between the unique sequence differences in the variable region of PFKFB3. As shown in Fig. 1B, two splicing variants of PFKFB3 were observed in the different human tumor cell lines studied. Amplified fragments from each PCR product were subcloned and sequenced, and we confirmed the exon pattern of the dominant isoform to consist of exons A, C, and G (henceforth termed PFKFB3-ACG). The structure of this isoform is consistent with that of the ubiquitous/placenta PFK-2 isoform previously reported (19, 20). None of splicing variants contain exons B, D, E, and F in these tumor cell lines, although these exons are expressed in human and rat brain (18). The lower molecular weight isoform contains only exons A and G (termed PFKFB3-AG), and this isoform also was detected in each of the cell lines examined. The isoform PFKFB3-ACDG was detected only in skeletal muscle. PFKFB3-AG was detected in human brain; however, its homologue was not described to be present among known rat PFKFB3 isoforms (22). Sequence analysis also confirmed that each isoform contained the COOH-terminal, variable region of PFKFB3 (data not shown).

We next examined the expression pattern of splicing variants of PFKFB3 in human colon carcinoma. Using primers specific for the PFKFB3 variable region, we examined the expression of PFKFB3 mRNA in five samples of colon carcinoma and corresponding normal tissue obtained from the same individuals. We observed high expression levels of PFKFB3-ACG mRNA in three of five individuals (Fig. 1C and D). The mean value of the intensity of PFKFB3-ACG expression relative to β-actin was 0.76 ± 0.29 in the carcinoma tissue and 0.38 ± 0.14 in normal tissue. Overall, PFKFB3-ACG expression was significantly higher in the carcinoma than in corresponding normal tissue ($P = 0.034$). Lower levels of expression of PFKFB3-AG mRNA were observed in both the tumor and normal colonic cDNA specimens (Fig. 1C). Although this analysis is semiquantitative, it suggests that among PFKFB3 isoforms, PFKFB3-ACG is induced more strongly at the transcriptional level in colonic epithelial cells than the PFKFB3-AG isoform. We next examined the expression of PFKFB3 mRNA in the same samples using quantitative, real-time PCR. PFKFB3 is distinguished from other PFKFB family members by the presence of an AU-rich, mRNA instability motif in the 3′ untranslated region of its mRNA (12), and we designed primers to amplify the region containing the AU-rich element. High



expression levels of PFKFB3 mRNA were confirmed in three of five samples of colon carcinoma (Fig. 1D). The mean values of the expression level of PFKFB3 were 2.72 ± 1.04 in carcinoma and 1.65 ± 0.64 in normal tissue. PFKFB3 expression was significantly higher in carcinoma than in normal tissue ($P = 0.049$). Further induction may be attributed to malignant transformation and to the metabolic demands for ATP and nucleotide precursors that are associated with cell proliferation (12).

Forced expression of PFKFB3 protein activates glycolysis and promotes cell proliferation. To confirm a regulatory role for PFKFB3 in cell proliferation (12, 17), we cloned mouse PFKFB3 and investigated the effect of its forced expression on the proliferation of COS-7 cells. We also confirmed by sequencing that this variant is homologous to human PFKFB3-ACG (93.6% identity in the variable regions). The contribution of the Ser^{461} to the change in the kinetic properties of PFK-2 induced by phosphorylation was studied by site-directed mutagenesis. Ser^{461} of PFKFB3-ACG was mutated into glutamate, which structurally mimics a phosphorylated serine, to create a PFKFB3-ACG^{S461E} protein. pcDNA3.1-PFKFB3-ACG (461WT) and PFKFB3-ACG^{S461E} (461E) was transfected into COS-7 cells and the expression of recombinant PFKFB3-ACG protein determined by Western blot analysis using anti-V5 antibody (which recognizes the short, amino acid fusion tag introduced into the protein by the cloning vector). Marked expression of recombinant PFKFB3-ACG protein was observed in both of 461WT- and the 461E-transfected COS-7 cells (Fig. 2A). The transfection efficiency was analyzed by β-Gal Staining Kit (Invitrogen), and 70% of COS-7 cells expressed reporter gene protein. We found that intracellular F2,6BP levels were markedly elevated in COS-7-461WT cells when compared with empty vector-transfected, COS-7 cells (Fig. 2B). Interestingly, intracellular F2,6BP levels were lower in COS-7-461E than in COS-7-461WT cells. Extracellular lactate levels in COS-7-461WT cell cultures also were increased, and a further increase was observed COS-7-461E cells, indicating an enhancement of glycolytic flux (Fig. 2C). Finally, COS7-461E cells showed an increase in proliferation when compared with control vector transfectants and COS-7-461WT cells (Fig. 2D). Taken together, these observations indicate that recombinantly expressed PFKFB3-ACG protein activates glycolytic flux via the synthesis of F2,6BP, and that phosphorylation of Ser^{461} is associated with a high glycolytic rate, leading to enhanced proliferation. We speculate that the lower level of F2,6BP in COS-7-461E indicates a negative feedback effect due to enhanced proliferation.

Oligomycin and serum induces the phosphorylation and activation of the PFKFB3 enzyme. Monocyte/macrophage activation is associated with the induction of glucose-dependent metabolic pathways (23, 26–28). Oligomycin is an inhibitor of oxidative phosphorylation that induces the phosphorylation of AMPK, which leads to the phosphorylation of PFKFB3 protein and enhanced glycolysis (23). We treated RAW 264.7 monocytes with oligomycin and characterized the expression and the phosphorylation of the PFKFB3 protein by anti-phospho-PFK-2(PFKFB3) immunoblotting. RAW 264.7 monocytes mainly express PFKFB3-ACG and

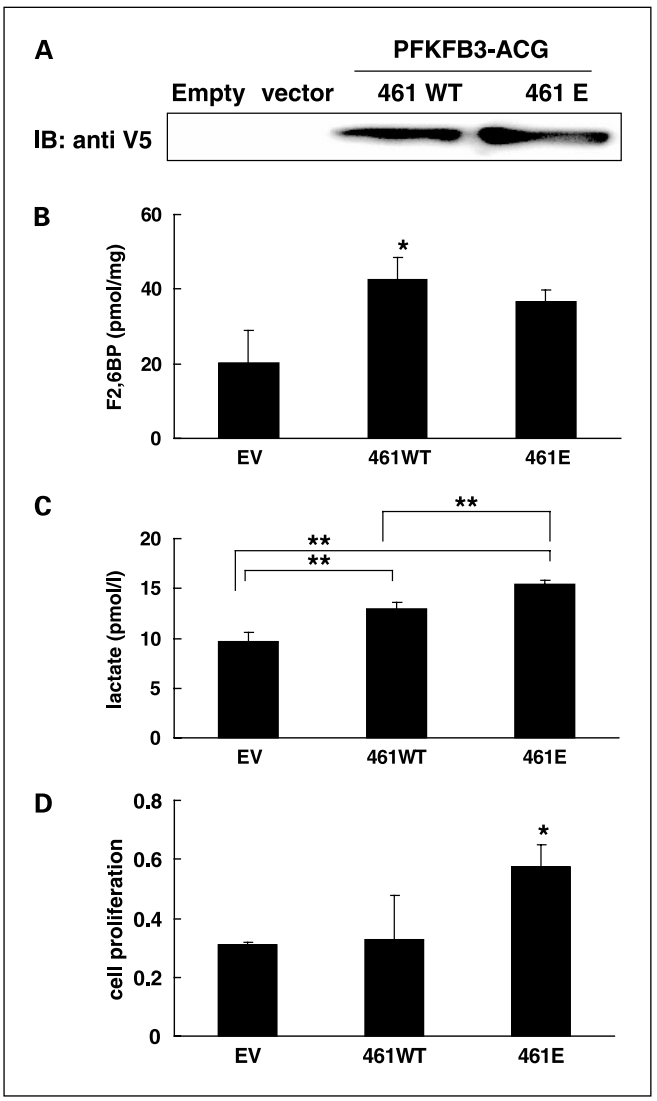


Fig. 2. The effect of wild-type PFKFB3-ACG (461WT) or PFKFB3-ACG^{S461E} (461E) expression on F2,6BP production, lactate secretion, and proliferation in COS-7 cells. *A*, recombinant PFKFB3 protein expression was verified by Western blot analysis using an anti-V5 antibody that recognizes the V5 peptide tag contained within the COOH-terminal region of the recombinant protein. Intracellular F2,6BP content (*B*), lactate in medium (*C*), and cell proliferation (*D*) was measured as described in Materials and Methods. *Columns,* means of four independent experiments; *bars,* ±SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).

Fig. 1. Reverse transcription-PCR analysis of the expression of mRNA for different PFKFB3 isoforms in human tumor cell lines. *A*, schematic diagram of the *PFKFB3* gene. Alternative splicing of the variable region consisting of exons 13 to 19 (A-G, *shaded*) leads to the expression of several isoforms in a tissue-specific manner. The AUUUA mRNA instability motif is encoded by exon 19 (exon G). The Ser^{461} phosphorylation site is located in exon 15 (exon C). *B*, exon pattern of the dominant isoform in human tumor cell lines consists of exons A, C, and G (termed PFKFB3-ACG). Total RNA was extracted from each cell line and cDNA was synthesized by reverse transcriptase. Specific primers to analyze the expression of different PFKFB3 isoforms were designed as described in Materials and Methods. The PCR products were separated by electrophoresis in 5% polyacrylamide gel and stained with ethidium bromide. Each amplified fragment then was sequenced as described in Materials and Methods. *C*, comparison of the expression of PFKFB3 mRNA in five human colon carcinoma and corresponding normal tissues. The expression of PFKFB3-ACG mRNA and PFKFB3-AG mRNA was determined by reverse transcription-PCR. *D*, densitometric analysis of PFKFB3-ACG expression in specimens of colon carcinoma and corresponding normal tissues (*left*). The expression of PFKFB3 mRNA was determined by quantitative, real-time PCR analysis for PFKFB3 (*right*).

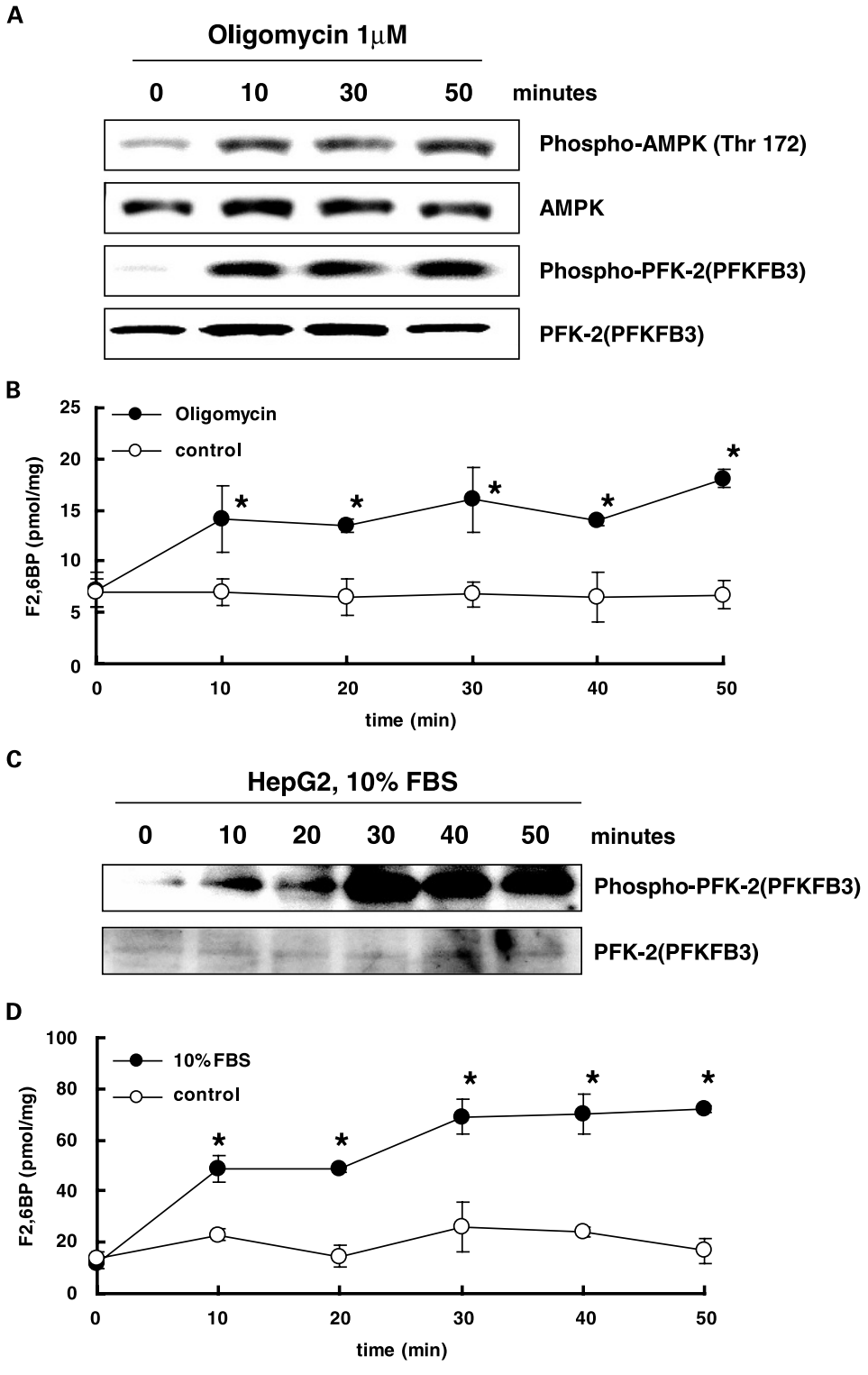


Fig. 3. Effect of oligomycin and serum on the phosphorylation of PFKFB3 protein. *A*, RAW 264.7 monocytes were treated with oligomycin for the time periods shown, and the phosphorylation and expression of AMPK protein were determined by Western blot analysis. Membranes were stripped and reprobed with anti-phospho-PFK-2(PFKFB3) antibody or anti- PFK-2(PFKFB3) antibody. *B*, intracellular F2,6BP content in RAW 264.7 cells was measured in oligomycin-treated cells as described in Materials and Methods. *Points,* means of three independent experiments; *bars,* ± SD. *, $P < 0.05$ (Student's *t* test). *C*, phosphorylation of PFKFB3 protein in response to serum stimulation of HepG2 cells. Serum-starved HepG2 cells were stimulated by FBS and the phosphorylation of PFKFB3 protein was determined by Western blot analysis using anti-phospho-PFK-2(PFKFB3) antibody. Membranes were stripped and reprobed with anti-PFK-2(PFKFB3) antibody. *D*, intracellular F2,6BP content in HepG2 cells was measured as described in Materials and Methods. *Points,* means of three independent experiments; *bars,* ±SD. *, $P < 0.05$ (Student's *t* test).

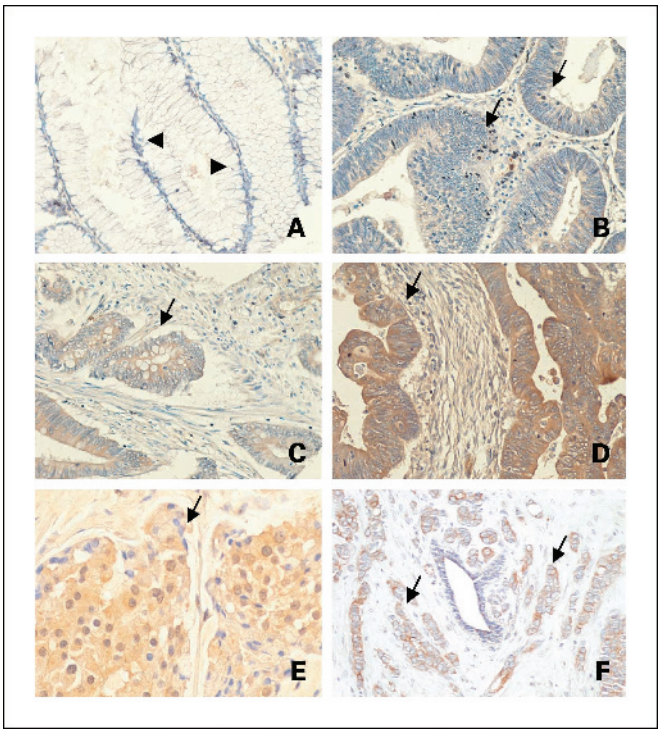


Fig. 4. Phosphorylation of PFKFB3 protein in human tumor tissue and corresponding normal tissue. *A*, immunohistochemical analysis was done with an anti-phospho-PFK-2(PFKFB3) antibody. Normal colon epithelium shows negative staining (*arrowheads*). *B-D*, colon carcinomas of various grades show positive staining: weakly positive (*B*), moderately positive (*C*), strongly positive (*D*). *E-F*, breast carcinoma stains positively: moderately positive (*E*), strongly positive (*F*). Each positive staining was indicated by arrows. Original magnification, ×200.

PFKFB3-AG, as assessed by primers specific for the PFKFB3 variable region (data not shown). The Ser^{461} phosphorylation site is located in the COOH-terminal, variable region of exon C and we considered that an anti-phospho-PFK-2(PFKFB3)-specific antibody may detect the phosphorylated PFKFB3-ACG protein in RAW 264.7 cells. Western blotting analysis confirmed the phosphorylation of AMPK (Thr^{172}) in response to oligomycin treatment, and total AMPK protein content was not affected (Fig. 3A). A single, strong band corresponding to the 62-kDa phosphorylated PFKFB3 protein was observed in oligomycin-treated but not in untreated RAW 264.7 monocytes (Fig. 3A). We also observed an increase in the intracellular content of F2,6BP after incubation with oligomycin (Fig. 3B), which is consistent overall with the induction of PFK-2 activity by oligomycin treatment.

Previous reports have indicated that intracellular F2,6BP levels also increase in response to serum stimulation (29, 30). We hypothesized that serum may increase the kinase activity of PFKFB3 protein via phosphorylation of the PFKFB3 Ser^{461} residue. Serum-starved HepG2 cells were exposed to serum and the phosphorylation of the PFKFB3 protein was determined by Western blot analysis using anti-phospho-PFK-2(PFKFB3) antibody. As shown in Fig. 3C, the phosphorylation of PFKFB3 was significantly increased by serum stimulation, without affecting PFKFB3 protein content. Intracellular F2,6BP content also was markedly increased by serum stimulation of HepG2 cells (Fig. 3D). Taken together, these observations indicate that the activation of glycolytic flux via the production of F2,6BP is associated with the phosphorylation of the PFKFB3 protein, as detected by an antibody directed against the phosphorylated PFKFB3 peptide, RRNSVTP.

PFKFB3 protein is highly phosphorylated in human cancers. In a previous report, high levels of PFKFB3 protein were observed in different human tumors (17). PFK-2/FBPase catalyzes both the synthesis and degradation of F2,6BP. PFKFB3 is

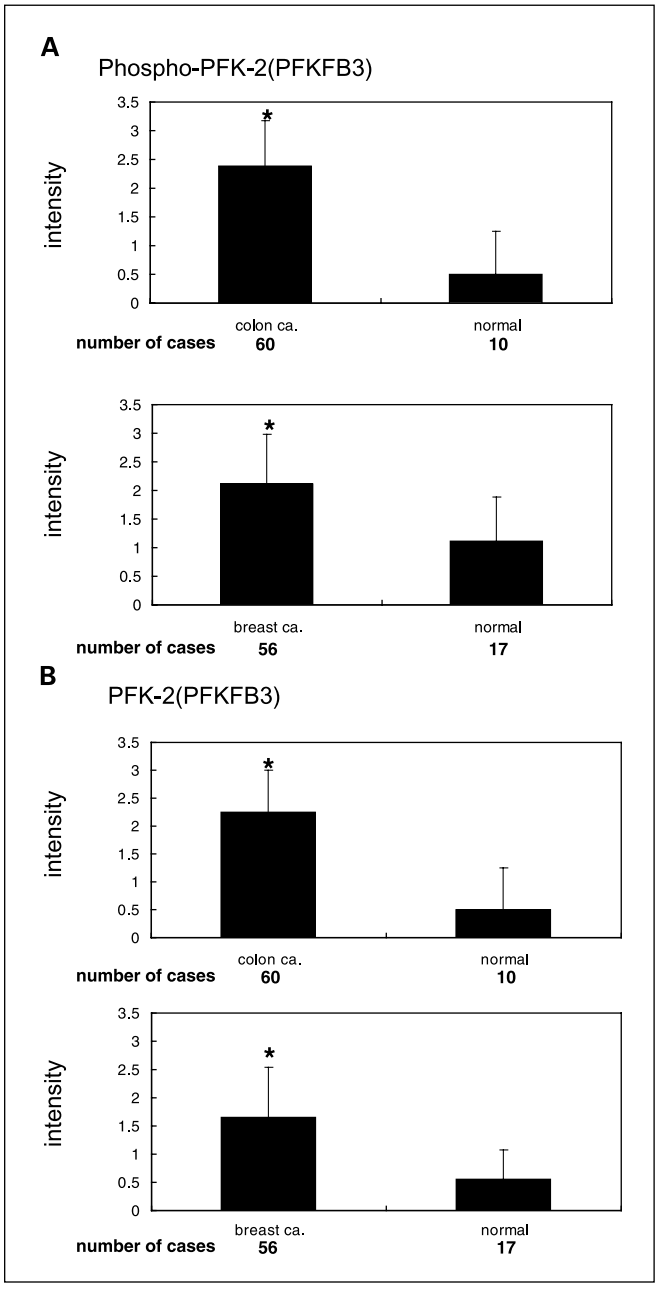


Fig. 5. Mean intensity scores of phosphorylation of PFKFB3 protein and immunoreactive PFKFB3 protein in human colon and breast carcinoma and corresponding normal tissues. *A*, mean intensity scores of phosphorylation of PFKFB3 protein in colon (*top*) and breast carcinoma (*bottom*). Immunohistochemistry was done using anti-phospho-PFK-2(PFKFB3) antibody. *B*, mean intensity scores of immunoreactive PFKFB3 protein in colon (*top*) and breast carcinoma (*bottom*). Immunohistochemistry was done using anti-PFK-2(PFKFB3) antibody. The intensity was graded as 0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive. *Columns,* means; *bars,* ±SD. *, $P < 0.05$ (Student's *t* test).

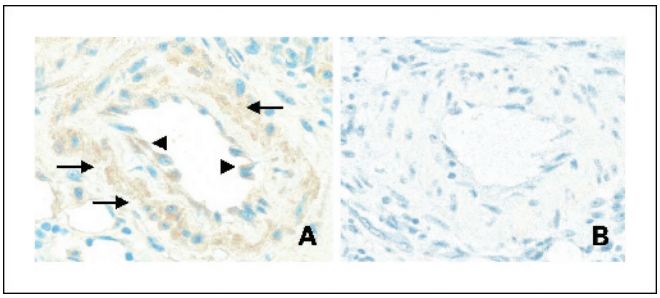


Fig. 6. The phosphorylation of PFKFB3 protein in vascular endothelial cells and smooth muscle cells. Immunohistochemistry was performed with an anti-phospho-PFK-2(PFKFB3) antibody. *A*, marked positive staining was observed in vascular endothelial cells (*arrowheads*) and smooth muscle cells (*arrows*). *B*, preimmune serum exhibited negative staining. Original magnification, ×400.

considered to have a high ratio of kinase to phosphatase activity, and the kinase activity of phosphorylated PFKFB3 is higher than that of the unphosphorylated PFKFB3 protein (23). To study the phosphorylation of the PFKFB3 protein in human tumors, 116 samples of human colon and breast carcinoma and 27 samples of corresponding normal tissue were analyzed by immunohistochemistry using anti-phospho-PFK-2(PFKFB3) antibody. As shown in Figs. 4 and 5, PFKFB3 protein was highly phosphorylated in colon and breast carcinoma when compared with epithelial cells in the corresponding, normal tissue types. Of note, phosphorylation of PFKFB3 protein was most readily detected in areas of carcinoma that were invading adjacent, normal tissue, which suggests that phosphorylation of PFKFB3 protein may be associated with the properties of tumor invasion, hypoxia, migration, or metastasis (Fig. 4F). High tissue levels of PFKFB3 protein also were confirmed by immunohistochemistry with anti-PFK-2(PFKFB3) antibody, and the distribution of PFKFB3-positive cell was virtually identical to that observed for phosphorylated PFKFB3 protein positivity. A tumor stage–dependent increase in phosphorylated PFKFB3 protein was not detected in the breast or colon specimens analyzed (data not shown). Finally, a prominent level of phosphorylated PFKFB3 protein was detected in vascular endothelial cells and in smooth muscle cells (Fig. 6). These findings suggest that the expression of PFK-2/PFKFB3 and its phosphorylation may contribute to tumor-associated angiogenesis.

Discussion

The accelerated glycolysis that is characteristic of transformed cells has been attributed to two mechanisms: the rapid growth rate and requirement for nucleotide precursors due to malignant transformation (31) and the hypoxic tissue microenvironment (32, 33). Under hypoxic condition, tumor cells must metabolize glucose anaerobically to generate ATP. Ras or Src oncogene-mediated transformation of fibroblasts *in vitro* leads to an up-regulation of glucose transporter mRNA and protein (34, 35). Recently, hypoxia-inducible factor was identified as a transcriptional factor that is necessary for the up-regulation of genes encoding glucose transporters and glycolytic enzymes (31, 36). F2,6BP is a potent, positive allosteric regulator of glycolysis, and its discovery has led to the realization that it plays an important role in glycolytic flux in rapidly proliferating cells. The *PFKFB3* gene responsible for the production of F2,6BP is under the transcriptional control of hypoxia-inducible factor-1 (36). In the present study, we provide evidence for high levels of phosphorylation of PFKFB3 protein in tumor cells, which is consistent for a role for this enzyme in the high glycolytic flux of human tumors.

The PFK-2/FBPase isoforms were originally named based on the tissue from which they were first purified, such as liver PFK-2 (PFKFB1), heart PFK-2 (PFKFB2), ubiquitous/placenta PFK-2 (PFKFB3), brain PFK-2 (PFKFB3), and testis PFK-2 (PFKFB4; refs. 3, 8, 20, 21). Differences in the kinetic properties of the PFK-2/FBPase family members are better explained by their gene origin however rather than their tissue distribution. The *PFKFB3* gene contains at least 19 exons, and alternative splicing of the COOH-terminal variable region generates multiple isoforms (18, 22). We analyzed the expression pattern of the COOH-terminal region of PFKFB3 and found that the PFKFB3-ACG isoform is the major one expressed in normal colonic epithelium and in colon carcinoma. The isoform PFKFB3-ACG was identified by sequencing to correspond to ubiquitous/placenta PFK-2 (15, 19, 20). We also provide evidence for the expression of an additional variant of PFKFB3, which has the structure of PFKFB3-AG.

Among the different members of the PFKFB family, PFKFB3 is considered to mediate the production of F2,6BP in proliferating cells because of its high, intrinsic kinase activity. Previous studies have supported the production of PFKFB3 protein in human tumor tissue (17). Although the core, catalytic structures of the different PFK-2/FBPase proteins are highly conserved, there are differences in the kinase and bisphosphatase properties of the different isoforms that are due in part to structural variations in the terminal regions. These bifunctional enzymes function as homodimers, and the terminal regions likely elicit their regulatory function by conformational changes induced by phosphorylation or by the binding of regulatory effectors (7). In the present study, we used a specific anti-phospho-PFK-2(PFKFB3) antibody to show that PFKFB3 protein is highly phosphorylated in human cancer tissue when compared with corresponding normal tissue. This finding suggests that the phosphorylation of PFKFB3 protein may play an important role in the activation of glycolysis in tumor tissue. The Ser^{461} within the PFKFB3 COOH-terminal region is a consensus site for the phosphorylation by AMPK (23). AMPK is considered important for the development of tolerance to nutrient starvation by pancreatic cancer cells (23, 37) and the activation of AMPK in cancer cells within anoxic areas may further stimulate glycolysis by phosphorylating the PFKFB3 protein. The expression of PFKFB3 protein also is induced in monocytes stimulated with lipopolysaccharide, and the protein is phosphorylated and activated by AMPK (23). The stimulation of monocyte glycolysis via the AMPK-induced phosphorylation and activation of PFKFB3 protein may be important for furnishing ATP to sustain inflammatory mediator production in sites of anaerobic infection.

The reliance of tumor cells on enhanced glycolysis for sustaining the metabolic and nucleotide precursor demands of a high proliferative rate has suggested a potential role for

glycolysis inhibition in cancer treatment. The recent report of a role for the glycolysis inhibitor, 2-deoxy-D-glucose, in increasing the antitumor efficacy of Adriamycin and paclitaxel *in vivo* has heightened further interest in this concept (38). We suggest that further investigation into the mechanisms responsible for the induction of PFKFB3 expression and Ser^{461} phosphorylation in transformed cells will provide new approaches for selective inhibition of tumor cell glycolysis.

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