Nuclear Targeting of 6-Phosphofructo-2-kinase (PFKFB3) Increases Proliferation via Cyclin-dependent Kinases*[s]

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The regulation of metabolism and growth must be tightly coupled to guarantee the efficient use of energy and anabolic substrates throughout the cell cycle. Fructose 2,6-bisphosphate (Fru-2,6-BP) is an allosteric activator of 6-phosphofructo-1-kinase (PFK-1), a rate-limiting enzyme and essential control point in glycolysis. The concentration of Fru-2,6-BP in mammalian cells is set by four 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB1–4), which interconvert fructose 6-phosphate and Fru-2,6-BP. The relative functions of the PFKFB3 and PFKFB4 enzymes are of particular interest because they are activated in human cancers and increased by mitogens and low oxygen. We examined the cellular localization of PFKFB3 and PFKFB4 and unexpectedly found that whereas PFKFB4 localized to the cytoplasm (*i.e.* the site of glycolysis), PFKFB3 localized to the nucleus. We then overexpressed PFKFB3 and observed no change in glucose metabolism but rather a marked increase in cell proliferation. These effects on proliferation were completely abrogated by mutating either the active site or nuclear localization residues of PFKFB3, demonstrating a requirement for nuclear delivery of Fru-2,6-BP. Using protein array analyses, we then found that ectopic expression of PFKFB3 increased the expression of several key cell cycle proteins, including cyclin-dependent kinase (Cdk)-1, Cdc25C, and cyclin D3 and decreased the expression of the cell cycle inhibitor p27, a universal inhibitor of Cdk-1 and the cell cycle. We also observed that the addition of Fru-2,6-BP to HeLa cell lysates increased the phosphorylation of the Cdk-specific Thr-187 site of p27. Taken together, these observations demonstrate an unexpected role for PFKFB3 in nuclear signaling and indicate that Fru-2,6-BP may couple the activation of glucose metabolism with cell proliferation.

Neoplastic transformation and growth require a massive increase in glucose uptake and glycolytic flux not only for energy production but also for the synthesis of nucleic acids, amino acids, and fatty acids. A central control point of glycolysis is the negative allosteric regulation of a rate-limiting enzyme, phosphofructokinase-1 (PFK-1),[2] by ATP (*i.e.* the Pasteur effect) (1, 2). When intracellular ATP production exceeds usage, ATP inhibits PFK-1 and glycolytic flux. Fructose 2,6-bisphosphate (Fru-2,6-BP) is a potent allosteric activator of PFK-1 that overrides this inhibitory influence of ATP on PFK-1, allowing forward flux of the entire pathway (3–5).

The steady-state cellular concentration of Fru-2,6-BP is dependent on the activities of bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB), which are encoded by four independent genes (*PFKFB1–4*) (6, 7). The PFKFB3 mRNA is distinguished by the presence of multiple copies of an AUUUA instability motif in its 3′-untranslated region and the PFKFB3 protein product has a high kinase:phosphatase activity ratio (740:1) (8). PFKFB3 mRNA is overexpressed by rapidly proliferating transformed cells and the PFKFB3 protein is highly expressed in solid tumors and leukemias (8–11). PFKFB3 expression is increased in response to several mitogenic stimuli, including progesterone, serum, and insulin (12–14). These studies indicate that the PFKFB3 enzyme may serve an essential function in the regulation of glucose metabolism during cell proliferation.

The PFKFB3 mRNA is spliced into several variants that encode distinct carboxyl-terminal domains (9, 15). Importantly, the functional consequences of the disparate carboxyl-terminal variants of PFKFB3 are unknown. The mRNA splice variant 5 is the dominant PFKFB3 mRNA in human brain, several transformed cells, and colon adenocarcinoma tissues (9, 10). In the following series of experiments, we present data that the carboxyl-terminal domain of PFKFB3 variant 5 localizes the enzyme to the nucleus where its product, Fru-2,6-BP, increases the expression and activity of cyclin-dependent kinase-1. These data demonstrate a heretofore unidentified function of the PFKFB3 enzyme that is distinct from glycolysis, and provide a potential mechanism for the coupling of metabolism and proliferation.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa, HCT116, and MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified

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[2] The abbreviations used are: PFK-1, 6-phosphofructo-1-kinase; Fru-2,6-BP, fructose 2,6-bisphosphate; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Cdk, cyclin-dependent kinase; EGFP, enhanced green fluorescent protein; RT, reverse transcriptase; GFP, green fluorescent protein; WT, wild type; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase.

Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Clontech) under 5% CO_2 at 37 °C.

Constructs, PCR, and RT-PCR—RNA isolation and cDNA synthesis were performed using commercial kits (Qiagen) following the manufacturer's instructions. All primers were obtained from Integrated DNA Technologies. PFKFB3 splice variants were amplified from brain first-strand cDNA (Clontech) using the following primers: 5′-GTCGCTTATGGCT-GCCGTGT-3′ (forward) and 5′-GCCAAGCATGGTTCTCT-TCG-3′ (reverse). All RT-PCR amplicons were sequenced to confirm the identity of the mRNA splice variants. PFKFB3 and p27 mRNA expression was determined by SYBR Green-based Real Time PCR analyses of cDNA synthesized. The primers used were as follows: PFKFB3, 5′-CAGTTGTGGCCTCCAA-TATC-3′ (forward) and 5′-GGCTTCATAGCAACTGATCC-3′ (reverse); p27, 5′-ACTTGGAGAAGCACTGCAGAGACA-3′ (forward) and 5′-TCTGTAGTAGAACTCGGGCAAG-CTG-3′ (reverse); β-actin, 5′-CCAACCGCGAGAAATGACC-3′ (forward) and 5′-GGAGTCCATCACGATGCCAG-3′ (reverse). FLAG-PFKFB3 containing the complete PFKFB3 coding sequence (GenBank[TM] accession number AF056320) and FLAG epitope at its NH_2 terminus was generated by PCR using brain cDNA as template and using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) for the amplification. The following primers were used: 5′-TAGGATCCATGGACTAC-AAGGACGACGACGACAAGTTGGAACTGACGCAGAG-CCGA-3′ (forward) and 5′-TGAAGCTTGGAAATGGAATG-GAACCGAC-3′ (reverse), which also had BamHI and HindIII restriction sites at NH_2- and COOH-terminals, respectively. A BamHI/HindIII FLAG-PFKFB3 expression cassette was subcloned into pAAV-MCS plasmid (Stratagene). The pEGFP-Link vector used for generating carboxyl-terminal GFP fusions was prepared by PCR amplification of the enhanced green fluorescent protein open reading frame from pEGFP1 (Clontech) with the following primers: 5′-GATCAAGCTTATGGACTAC-AAGGACGACGACGACAACGTGAGCAAGGGCGAGGA-3′ (forward) and 5′-GCGGATCCAGAACCAGACTTGTACAG-CTCGTCCAT-3′ (reverse). The resulting PCR product was then digested with HindIII and BamHI and subcloned into the corresponding sites in pcDNA3 to generate pEGFP-Link, which encodes EGFP with an NH_2-terminal FLAG epitope tag. To produce chimeric EGFP proteins, each of the five cloned splice variants from brain was amplified using primers: 5′-TCTG-GATCCGTCTGCACACACCGGGA-3′ and 5′-CATTCTAG-AAGTCCTCAGGATACGTTTTG-3′. The resulting PCR products were then digested with BamHI and XbaI and subcloned into the corresponding sites of pEGFP-Link to create five constructs: pEGFP-PFKFB3-Cterm (V1, V3, V4, V5, and V6).

The pIRESneo3-His$_6$-PFKFB3 mammalian expression vector was derived using a bacterial expression vector as an intermediate to obtain an NH_2-terminal His$_6$ tag. Briefly, the entire open reading frame for PFKFB3 was amplified from human first-strand lung cDNA (Clontech) with the following primers: 5′-GACGACGACAAGATGCCGTTGGAACTGAC-3′ (forward) and 5′-GAGGAGAAGCCCGGTGCCAAGCATGGTT-CTCT-3′. The resulting PCR product was then cloned into the pET-30 Ek/LIC bacterial expression vector (Novagen) accord-

ing to the protocol for ligation-independent cloning to create pET30Ek/Lic-PFKFB3. The resulting construct was then used as a template for PCR amplification with the following primers: 5′-GTTGAATTCACCATGCACCATCATCATCATCATTC-TTCTG-3′ (forward) and 5′-GTAGCGGCCGCAGTCCTCA-GGATACGTTTTG-3′ (reverse). The PCR product was digested with EcoRI and NotI and subcloned in corresponding sites in the pIRESneo3 mammalian expression vector to yield pIRESneo3-His$_6$-PFKFB3, which retained the NH_2-terminal His$_6$ tag and other NH_2-terminal linker features (*e.g.* S-tag) present in the pET-30 vector.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the QuikChange® II XL Site-directed Mutagenesis Kit (Stratagene) to generate stop codons in the carboxyl-terminal encoding region of FLAG- and His$_6$-PFKFB3. The following primer pairs were used to produce the indicated mutations, which were all verified by DNA sequencing: L502X, 5′-GGAGGTGCCCACGCAGTAACCTGGACAAAACA-3′ (forward) and 5′-CCTTTCATGTTTTGTCCAGGTTACTG-CGTGGGC-3′ (reverse); F479X, 5′-CCTCGCATCAACAGC-TAAGAGGAGCATGTGGCC-3′ (forward) and 5′-GGCCACA-TGCTCCTCTTAGCTGTTGATGCGAGG-3′ (reverse); K472X, 5′-CCCCGAACCCACCTAAAAGCCTCGCATCAAC-3′ (forward) and 5′-GTTGATGCGAGGCTTTTAGGTGGGTT-CGGGG-3′ (reverse); and R458X, 5′-GACCTAACCCGCTC-ATGTAACGCAATAGTGTC-3′ (forward) and 5′-GGGGTG-ACACTATTGCGTTACATGAGCGGGTT-3′ (reverse). The cytoplasmic mutant K472A/K473A was created using the following primers: 5′-CCCCGAACCCACCGCAGCACCTCGC-ATCAACAGCT-3′ (forward) and 5′-AGCTGTTGATGCGA-GGTGCTGCGGTGGGTTCGGGG-3′ (reverse). The kinase-inactive R75A/R76A mutant was created using the following primers: 5′-GTTCAACGTCGGGGAGTATGCAGCCGAGG-CTGTGAAGCAGTAC-3′ (forward) and 5′-GTACTGCT-TCACAGCCTCGGCTGCATACTCCCCGACGTTGAAC-3′ (reverse).

Transfections—HeLa cells were grown in collagen-coated plates and transient transfection of the plasmid constructs was achieved with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. For immunofluorescence studies, cells were grown on fibrinogen-coated coverslips and transfected with FuGENE 6 transfection reagent (Clontech).

[³H]Thymidine Incorporation—Thymidine incorporation experiments were conducted as described (16). 36 h after transfection with the PFKFB3 constructs, cells were pulsed with [*methyl*-³H]thymidine (1 µCi/ml) for 2 h. The cells were then washed with PBS and fixed in 5% trichloroacetic acid for 5 min and then washed twice in 5% trichloroacetic acid. The acid-insoluble material was dissolved in 2 N NaOH and counted for radioactivity by liquid scintillation counter.

Confocal/Fluorescence Microscopy—Transfected cells grown on coverslips were fixed in paraformaldehyde and processed as described previously (17). The wild type and mutant PFKFB3 forms that were expressed from the pIRESneo3–6XHis construct were detected with Alexa Fluor® 488-labeled Penta·His[TM] monoclonal antibody (Qiagen). HeLa cells that were transfected with PFKFB3 COOH-terminal GFP fusions were directly examined for the localization of GFP under fluo-

rescence. Immunofluorescence images were acquired with Zeiss Axiovert 200 microscope. To visualize FLAG-PFKFB3 (splice variant 5) expression by confocal microscopy, cells were incubated with anti-FLAG® rabbit polyclonal primary antibody (Sigma) and then with an Alexa Fluor® 488-labeled secondary antibody (Molecular Probes), each for 1 h. Slides were analyzed with a FluoView 500 confocal laser microscope (Olympus).

Cytoplasmic and Nuclear Fractionation—Nuclear and cytosolic fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce). The extraction procedure was conducted according to the manufacturer's protocol with minor modifications. The nuclear pellet was first briefly lysed in a protein lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, and 0.5% Triton X-100) and then immediately denatured in 2× Laemmli Buffer containing β-mercaptoethanol (final concentration, 1×). Before boiling, lysate was thinned using first 22-gauge and then 28-gauge needles.

Western Blot—For preparation of total cell lysates, cells were washed in cold PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, and 0.5% Triton X-100) containing protease and phosphatase inhibitors (Sigma). Samples were separated through a 12.5% SDS-PAGE gel under reducing conditions, and proteins were then transferred to Immobilon P membranes. Mouse polyclonal and monoclonal PFKFB3 antibodies raised against the COOH-terminal domain were obtained from Novus Biologicals (number H00005209-A01 and H00005209-M08). Mouse monoclonal anti-α-tubulin was purchased from Neo-Markers. Rabbit polyclonal anti-PFKFB4, PFKFB2, and PFK-1 were purchased from Abgent (PFKFB4, number AP8154c; PFKFB2, number AP8146a; PFK-1, number AP8135b). Rabbit polyclonal anti-Oct-1 was purchased from Santa Cruz Biotechnology. Rabbit polyclonals anti-Rb, phospho(Ser-780)-Rb, phospho(Ser-795)-Rb, Cdc25C, Cdk1, mouse monoclonal anti-cyclin D3, and horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG antibodies were purchased from Cell Signaling Technology. Antibodies specific for total and phospho(Thr-187)-p27 were purchased from Calbiochem. Mouse monoclonals anti-FLAG and anti-β-actin were purchased from Sigma. For detection of immunoreactive bands, ECL and ECL Plus were used (Amersham Biosciences).

Cell Cycle Antibody Array—Antibody array on total lysates obtained from cells transfected with either empty vector (control) or a construct carrying PFKFB3 (pIRESneo3–6XHis-PFKFB3) was performed by Full Moon BioSystems, Inc. (Sunnyvale, CA). The assay is based on the incubation of biotin-labeled total cell lysates with array slides on which antibodies are immobilized and the detection of the biotin-labeled protein-antibody complexes by Cy3-Streptavidin.

Cell Cycle Synchronization—HeLa cells were synchronized at the G_1/S boundary by double-thymidine block as previously described (18, 19). Exponentially growing cells were arrested at the G_1/S boundary by treatment with thymidine (2 mM) for 17 h, and were released from the arrest by washing twice with fresh medium. Cells were grown in fresh medium for 9 h and then re-treated with thymidine (2 mM) for 15 h. Cells were then released from the second block simply by replacing the thymi-

dine containing media with fresh growth media. Cells enriched in S, G_2/M, and G_1 phases were collected at 3, 7, and 12 h after release from the block, respectively.

Fru-2,6-BP Assay—Intracellular Fru-2,6-BP concentration was determined as previously described (20). Briefly, cells were centrifuged at $200 \times g$, resuspended in 20 volumes of 0.05 N NaOH, and then 1 volume of 0.1 N NaOH to obtain a pH > 11, vortexed for 10 s, incubated at 80 °C for 5 min, and cooled in an ice bath. Cell extracts were neutralized to pH 7.2 with ice-cold acetic acid in the presence of 20 mM Hepes. Samples were incubated at 25 °C for 2 min in the following assay mixture: 50 mM Tris, 2 mM Mg^{2+}, 1 mM Fru-6-P, 0.15 mM NAD, 10 units/liter PP_i-dependent PFK1, 0.45 kilounit/liter aldolase, 5 kilounit/liter triose-phosphate isomerase, and 1.7 kilounit/liter glycerol-3-phoshate dehydrogenase (Sigma). 0.5 mM Pyrophosphate was added and the rate of change in absorbance (OD = 339 nm) per min was followed for 5 min. Fru-2,6-BP was calculated based on a calibration curve produced by measuring 0.1 to 1 pmol of Fru-2,6-BP (Sigma) and normalized to total cellular protein.

Spectrophotometry—Concentrations of glucose and lactate in media were determined spectrophotometrically using the Glucose Assay Kit (Sigma) and Lactate Assay Kit (Trinity Biotech), respectively, following the manufacturer's instructions.

One-dimensional Nuclear Magnetic Resonance Spectroscopy—NMR analysis was carried out as described (21, 22). Briefly, cells were grown in medium containing uniformly labeled [^{13}C]glucose (2 g/liter) (Cambridge Isotopes Laboratories). Medium was frozen in liquid nitrogen and extracted with 10% trichloroacetic acid and lyophilized. Dried extract samples were redissolved in 100% D_2O. NMR spectra were recorded at 800 MHz on a Varian Inova Spectrometer. Spectra were obtained at 20 °C using PRESAT with a 90° excitation pulse with an acquisition time of 2 s and a recycle time of 5 s. Spectra were processed with 0.5 Hz line broadening and an unshifted Gaussian window function. Concentrations were determined by reference to internal 2,2-dimethyl-2-silapentane-5-sulfonate sodium present at a known concentration, and normalized to cell numbers. The concentrations of [^{13}C]glucose, [^{12}C]- and [^{13}C]lactate, [^{12}C]- and [^{13}C]alanine, [^{12}C]Thr, and [^{12}C]Val were determined by integration of the H-1 resonance of glucose and the methyl resonances of the other metabolites, respectively. Glucose consumption was determined from the difference in the concentration of [^{13}C]glucose at zero time and the end of the experiment (48 h). The enrichment into lactate and alanine was determined from the area of the ^{13}C-satellite peaks of the methyl resonances compared with the unlabeled species as described elsewhere (21): $F = [[^{13}C]Lac]/([[^{13}C]Lac] + [[^{12}C]Lac])$, where F is the fraction of glucose consumed that is converted to secreted lactate. The NMR experiments were done by the NMR Core of the Structural Biology Program at the University of Louisville James Graham Brown Cancer Center.

Bacterial Expression and in Vitro Kinase Assay—The PFKFB3 encoding region was PCR amplified from the pET30Ek/Lic-PFKFB3 vector using the following primer pair: 5′-CTTCATATGCCGTTGGAACTGACGCA-3′ and 5′-CTTCTCGAGGTGTTTCCTGGAGGAGTCAGC-3′. The resultant 1.56-kb PCR product was then digested with XhoI and NdeI and ligated into the corresponding sites within the

pET30b(+) vector. The final construct, pET30b(+)-PFKFB3, encoded full-length PFKFB3 with a COOH-terminal His$_6$ tag. The K472A/K473A substitution was then introduced into this parent construct using the same primers described above for site-directed mutagenesis. Both parent and double mutant (K472A/K473A) constructs were then transformed into the BL21 strain of *Escherichia coli*. One-liter cultures of both transformed strains in LB media were shaken for 16 h at 37 °C. The temperature was then reduced to 30 °C and an additional liter of fresh LB containing 2 mM isopropyl 1-thio-β-D-galactopyranoside was added to both cultures for induction in 1 mM isopropyl 1-thio-β-D-galactopyranoside. After 4 h of induction at 30 °C, cells were pelleted, washed once with PBS, and resuspended in 8 ml/pellet g wet weight of lysis buffer as described in the Qiagen protocol for native purification of bacterial expressed proteins with 5 mM β-mercaptoethanol. The resuspended bacterial cells were then lysed by sonication, and the lysate was clarified by centrifugation. The clarified lysate was then mixed with 6 ml of nickel-nitrilotriacetic acid-agarose equilibrated in His$_6$ wash buffer and purified according to the Qiagen protocol.

The reaction conditions for the enzymatic assay for wild type and K472A/K473A PFKFB3 were as described elsewhere except that a coupled enzyme assay system was employed to detect the Fru-2,6-BP (23). Briefly, 5 μg of purified enzyme was added to a final 50-μl reaction containing 100 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 0.1 mM EDTA, 5 mM ATP, 1 mM Fru-6-P, 5 mM potassium phosphate (KH$_2$PO$_4$), and 10 mM MgCl$_2$. The reaction was then incubated at 30 °C for 40 min, and the reaction was terminated by placing the reactions on ice. The reactions were then centrifuged through a Centricon-30 filter (Millipore) and the filtrate was assayed for Fru-2,6-BP as described above.

In Vitro 6-Phosphofructo-2-kinase Assay on Purified Nuclei—Nuclei for in vitro 6-phosphofructo-2-kinase activity were isolated from HeLa cells using a Nuclei Isolation Kit (Sigma, number NUC-101) following the manufacturer's instructions. The purity of nuclei was confirmed to be over 95% based on trypan blue staining. The following assay was developed to measure the generation of Fru-2,6-BP by relatively intact pure nuclei. Approximately 1×10^7 nuclei were incubated at 30 °C for 10, 20, and 30 min while shaking in a 200-μl kinase buffer containing 100 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.1 mM EDTA, 5 mM ATP, 1 mM Fru-6-P, 5 mM KH$_2$PO$_4$, and 10 mM MgCl$_2$. Aliquots from supernatant were used to measure Fru-2,6-BP production (see "Fru-2,6-BP Assay" above). A no substrate (Fru-6-P) control was included to correct for background signal.

Phosphorylation of p27 in Total Lysates—The *E. coli*-expressed recombinant full-length human p27 with the NH$_2$-terminal GST tag was obtained from SignalChem (number C28-30G-50). Immobilized Glutathione SwellGel Discs, spin cups, and collection tubes were purchased from Pierce Biotechnology (number 89855). Fru-2,6-BP (Sigma, number F7006) was dissolved in 10 mM NaOH for use.

Exponentially growing HeLa cells were washed in cold PBS and proteins were extracted with lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl$_2$, 1% Nonidet P-40, 1 mM

dithiothreitol, and 5% glycerol) containing protease inhibitors (Sigma). 3.5 μg of GST-p27 ($\sim$40 μl) was absorbed onto a glutathione disc in a spin column and then 2 mg of lysate was added (total volume of 600 μl). NaOH (vehicle control) or Fru-2,6-BP was immediately added onto the lysate (final concentration of 200 μM). Columns were incubated/rotated at room temperature for 25 min. Glutathione beads were then centrifuged and washed three times with PBS. Proteins were eluted under denaturing conditions using Laemmli buffer and subjected to Western blot analysis for phosphorylated and total p27.

RESULTS

PFKFB3 mRNA Splice Variant 5 Localizes to the Nucleus—PFKFB3 splice variant 5 previously was found to be the dominantly expressed splice variant in transformed cells (9, 10). We confirmed that HeLa, HCT116, and MDA-MB-231 transformed cells expressed the PFKFB3 splice variant 5 and, to a far lesser extent, splice variant 6, and did not express variants 1–4 using RT-PCR analyses (data not shown).

The six human PFKFB3 mRNA splice variants differ only at their carboxyl-terminal domains (24). We hypothesized that these carboxyl-terminal domains may confer differential subcellular trafficking of the PFKFB3 protein. A full-length FLAG-tagged PFKFB3 splice variant 5 construct was created using brain cDNA as template and transfected into HeLa cells. The subcellular localization was examined by confocal microscopy. Surprisingly, we observed that PFKFB3 splice variant 5 localized to the nucleus (Fig. 1*A*). Next, we examined endogenous PFKFB3 protein expression in cytoplasmic and nuclear fractions extracted from HeLa, HCT116, and MDA-MB-231 cells using a monoclonal antibody that recognizes the variable carboxyl terminus present in the dominantly expressed splice variant and found that PFKFB3 immunoreactive bands only were detected in nuclear fractions (Fig. 1*B*). We also probed the membranes with antibodies specific for PFKFB2 and PFKFB4 enzymes, which we previously demonstrated to be expressed at mRNA levels in these cell lines (data not shown) and with an antibody specific for the rate-limiting enzyme of glycolysis, PFK-1. As seen, PFKFB2, PFKFB4, and PFK-1 localize to the cytoplasm, the site of glycolysis. The purity of the cytoplasmic and nuclear fractions was confirmed with antibodies to α-tubulin and Oct-1, respectively. This finding is consistent with the immunofluorescence data obtained with ectopically expressed PFKFB3 and confirms that PFKFB3 localizes to the nucleus.

To examine the requirement of the alternatively spliced carboxyl-terminal domains for subcellular localization specificity, we coupled each carboxyl-terminal domain to GFP and ectopically expressed the fusion protein products in HeLa cells. Immunofluorescence analyses revealed GFP fusion protein localization patterns consistent with high nuclear localization of splice variants 4 and 5 (supplemental Fig. S1, V4 and V5). These findings suggest that the alternative carboxyl-terminal domains may function to differentially localize PFKFB3 variants.

We truncated the carboxyl-terminal domain of full-length PFKFB3 splice variant 5 using site-directed mutagenesis to determine the residues required for the trafficking of
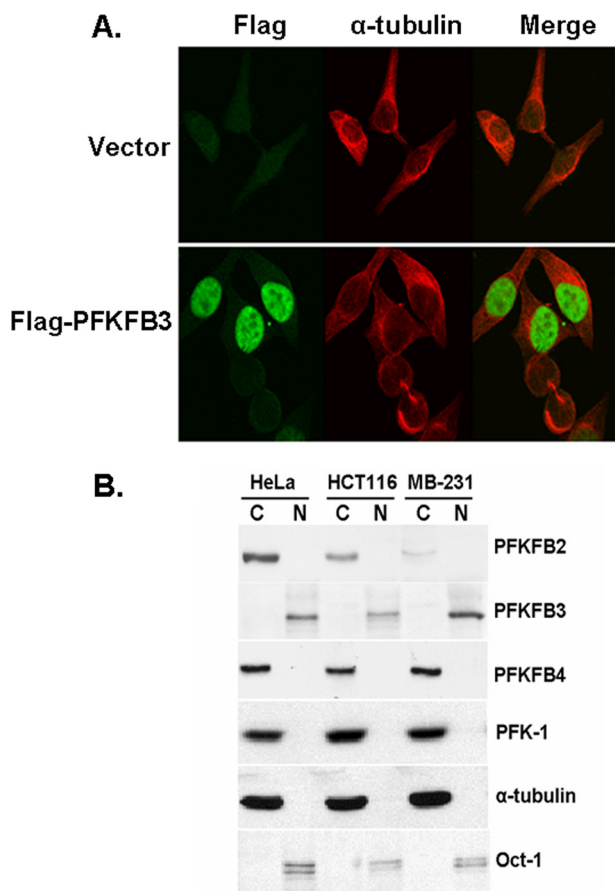
FIGURE 1. PFKFB3 localizes to the nucleus. *A*, HeLa cells were plated onto chamber slides and transfected with either empty vector (vector) or a construct carrying FLAG-tagged PFKFB3 (*Flag-PFKFB3*) and 24 h later, cells were fixed in paraformaldehyde and incubated with mouse anti-FLAG primary and then with Alexa Fluor® 488-labeled secondary antibodies. Slides were analyzed with a confocal laser microscope. α-Tubulin was used as cytoplasmic marker. *B*, cytoplasmic (*C*) and nuclear fractions (*N*) were prepared from the indicated cell lines and subjected to Western blot using a PFKFB3 carboxyl terminus specific antibody. Membranes were also probed with antibodies against PFK-1, PFKFB2, and PFKFB4. The purity of cytoplasmic and nuclear fractions was confirmed by assessing the expression of α-tubulin and Oct-1 proteins, respectively.

FIGURE 2. Nuclear localization of PFKFB3 mRNA splice variant 5 requires lysines 472 and 473. *A*, stop codons were introduced at the indicated positions *below* (*) in the construct carrying the full-length, His-tagged PFKFB3 splice variant 5 using site-directed mutagenesis and the resultant constructs were transfected into HeLa cells, which were then fixed and stained with a fluorescein isothiocyanate-conjugated monoclonal antibody against the His$_6$. *L502X*, PFKFB3-DAKKGPNPLMRRNSVTPLASPEPTKKPRINSFEEHVASTS-AALPSCLPPEVPTQ*; *F479X*, PFKFB3-DAKKGPNPLMRRNSVTPLASPEPTKKPRINS*; *K472X*, PFKFB3-DAKKGPNPLMRRNSVTPLASPEPT*; *R458X*, PFKFB3-DAKKGPN-PLM*. *B*, site-directed mutagenesis was used to substitute codons encoding lysines at 472 and 473 with codons encoding alanines using the construct containing the full-length, FLAG-tagged PFKFB3 splice variant 5. HeLa cells were transfected with constructs that encode either WT or mutant (K472A/K473A) PFKFB3 constructs and 24 h later cells were fixed in paraformaldehyde and incubated with a FLAG primary and then with Alexa Fluor® 488-labeled secondary antibodies. Images were captured under a confocal laser microscope. α-Tubulin was used as cytoplasmic marker. * indicates that residues afterwards are deleted.

PFKFB3 to the nucleus. We found that residues COOH-terminal of threonine 471 were required for the trafficking of the protein to the nucleus (Fig. 2*A*, compare F479X to K472X). This region contains the residue sequence KKPR, which fits a known nuclear localization signal consensus sequence K(K/R)*X*(R/K) (25) and we thus examined the requirement of lysines 472 and 473 for nuclear localization by confocal microscopy. We found that full-length PFKFB3 splice variant 5 localized to the cytoplasm if lysines 472 and 473 were substituted with alanines (K472A/K473A) (Fig. 2*B*, α-tubulin was used as a cytoplasmic marker). These findings suggest that PFKFB3 localizes to the nucleus and that the residues required for nuclear trafficking reside in the carboxyl terminus.

Ectopic Expression of PFKFB3 Variant 5 Does Not Increase Glycolytic Activity—We next transfected HeLa cells with constructs carrying either FLAG-tagged wild type (WT) PFKFB variant 5 (hereafter termed PFKFB3) or the cytoplasmic mutant PFKFB3 variant 5 (hereafter termed K472A/K473A PFKFB3),
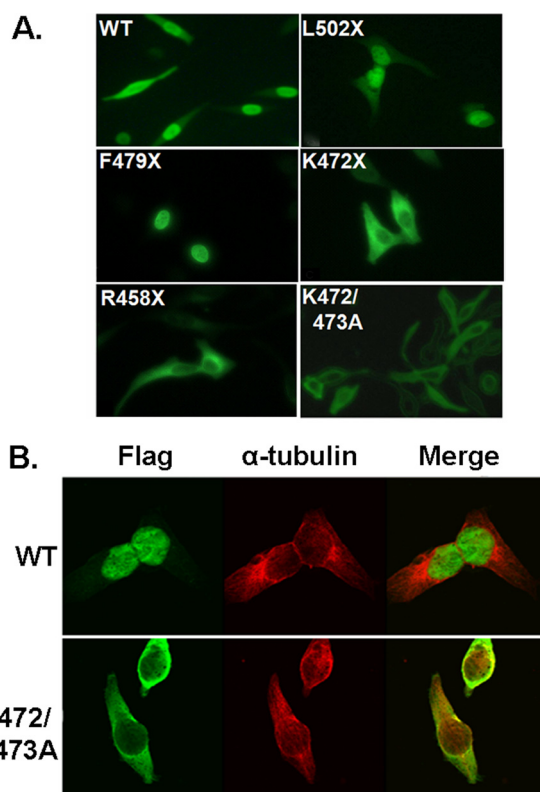
or with empty vector as a control. After 48 h, we confirmed that ectopic expression of the WT and K472A/K473A PFKFB3 was similar by Western blot analysis (Fig. 3*A*). A large increase in the steady-state concentration of Fru-2,6-BP was observed with the introduction of WT PFKFB3 and the 472/473A PFKFB3 mutant caused a further increase in Fru-2,6-BP production (Fig. 3*B*; Vector, 3.6 ± 0.4; WT, 35 ± 5; K472A/K473A, 82 ± 9 pmol/mg protein). To confirm that the observed increase in activity with the K472A/K473A mutant was due to changes in subcellular localization and not changes in the intrinsic enzymatic activity, we produced *E. coli* recombinant WT and K472A/K473A PFKFB3 protein and confirmed that the 6-phosphofructo-2-kinase activities were not significantly different (data not shown). We can only speculate that the increased *in vivo* activity obtained with the cytoplasmic localization of PFKFB3 may be due to increased substrate (Fru-6-P) availability in the cytoplasm. We were unable to detect Fru-2,6-BP in purified nuclei, which is presumably due to its loss through
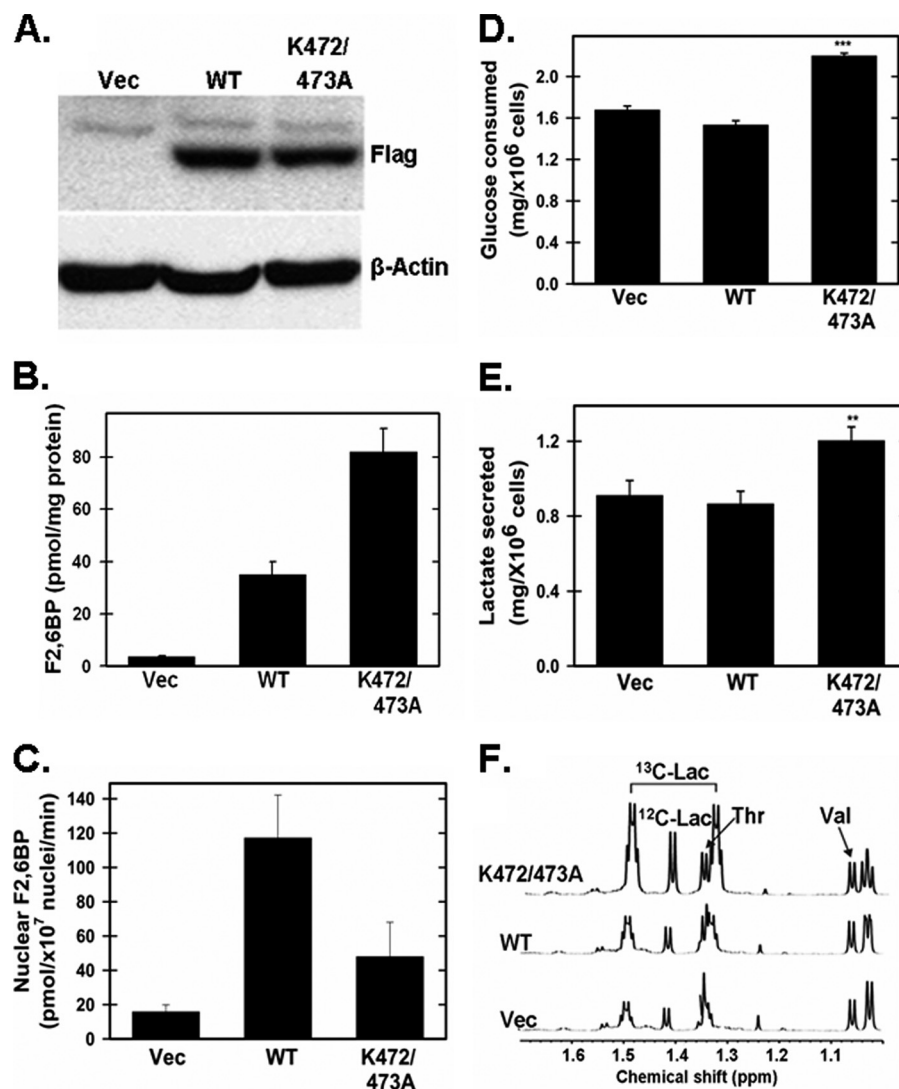
FIGURE 3. **Ectopic expression of wild type PFKFB3 variant 5 does not activate glycolysis.** HeLa cells were transfected with either WT or cytoplasmic mutant (K472A/K473A) FLAG-tagged PFKFB3 constructs, or with empty vector (*Vec*) as control. 48 h later (*A*) cells were lysed and subjected to Western blot using antibody specific for FLAG epitope; *B*, cells were lysed in NaOH and analyzed for total Fru-2,6-BP levels; *C*, nuclei were isolated and incubated in a kinase buffer (see "Experimental Procedures") for 10, 20, and 40 min, and supernatants were assayed for Fru-2,6-BP levels; *D* and *E*, media was collected and analyzed for glucose and lactate. Measurements were done in triplicate, and results are presented as mean ± S.D., $p < 0.01$; ***, $p < 0.001$. *F*, for one-dimensional NMR analysis, cells were transfected with the above constructs and grown in [^{13}C]glucose containing medium and 48 h later, medium was trichloroacetic acid-extracted, lyophilized, and dissolved in 100% D_2O. NMR spectra were recorded at 800 MHz on a Varian Inova Spectrometer. Shown are the methyl regions of representative samples.

nuclear pores during the purification procedure. However, we were able to measure 6-phosphofructo-2-kinase activity in the purified nuclei. Nuclei isolated from cells transfected with WT PFKFB3 exhibited 6-phosphofructo-2-kinase activity that was significantly higher than the nuclei obtained from both vector and K472A/K473A-transfected cells (Fig. 3*C*). A substantial amount of Fru-2,6-BP also was detected with the K472A/K473A mutant. This may be due to a partial nuclear localization of this mutant, which was observed in Western blot analyses (data not shown) and also, due to partial disruption of the nuclear membrane barrier during the isolation procedure. These findings demonstrate that PFKFB3 localizes to the nucleus and has the ability to produce Fru-2,6-BP in the nucleus.

We examined the supernatants of the transfected cells for glucose and lactate and found that only the K472A/K473A PFKFB3 mutant caused a significant increase in glucose consumption and total lactate secretion compared with cells transfected with empty vector and WT PFKFB3 (Fig. 3, *D* and *E*). Similar results were obtained in HCT116 cells (data not shown). Total lactate levels in medium may not be a reliable indicator of glycolytic activity, as other sources and pathways (*e.g.* glutamine and the pentose phosphate pathway) can contribute to lactate formation (26, 27). Therefore we examined the direct channeling of fully labeled [^{13}C]glucose to lactate using one-dimensional NMR spectroscopy. The enrichment of glucose into ^{13}C-labeled lactate and alanine were determined from the area of the ^{13}C-satellite peaks of the methyl resonances compared with the unlabeled species (21, 22) as described under "Experimental Procedures." Fig. 3*F* shows the methyl region of three representative samples (Vector, WT, and K472A/K473A). The relative intensity of the [^{13}C]lactate compared with that of the control valine residues ([^{13}C]lactate/valine) was highest in K472A/K473A (2.8-fold compared with vector), whereas [^{13}C]lactate/valine for the WT PFKFB3 was only 1.2-fold compared with vector. As a better indicator of glycolytic flux, the percentage of glucose consumed that is converted to lactate in the vector, WT, and K472A/K473A samples were 15 ± 2, 18 ± 2, and

31 ± 3, respectively. Collectively, these analyses demonstrate that expression of WT PFKFB3 in the nucleus causes no significant change in glycolytic flux, whereas the expression of PFKFB3 in the cytoplasm significantly stimulates glycolysis. That endogenous PFKFB3 appears to be predominantly expressed in the nucleus (in the three cell lines examined; Fig. 1*B*), suggests that this enzyme may have a function that is distinct from the regulation of glycolysis.

PFKFB3 Stimulates Cell Proliferation in the Nucleus—To examine the requirement of the kinase activity of PFKFB3 for a possible novel function in the nucleus, we created a mutant of PFKFB3 that is deficient in kinase activity, *i.e.* Fru-2,6-BP production. The recently published crystal structure of PFKFB3 (28) predicts the requirement of five arginine residues for con-

tact with the substrate, Fru-6-P (Arg-75, Arg-76, Arg-98, Arg-132, and Arg-189). Using the full-length, FLAG-tagged PFKFB3 construct as template, two consecutive arginines (Arg-75 and Arg-76) were substituted with alanines by site-directed mutagenesis and the resulting construct (termed R75/76A) was tested for its ability to produce Fru-2,6-BP *in vivo* by transfection into HeLa cells followed by measurement of Fru-2,6-BP levels in total lysates. Kinase activity of the R75A/R76A mutant was abolished (supplemental Fig. S2*A*). We also confirmed that these substitutions did not change subcellular localization of PFKFB3 (supplemental Fig. S2*B*). HeLa cells were transfected with WT PFKFB3, cytoplasmic (K472A/K473A), or kinase-inactive (R75A/R76A) mutant constructs, or with empty vector as control, and 48 h later, cell proliferation was determined by viable cell enumeration with trypan blue exclusion on a hemacytometer and [^{3}H]thymidine incorporation. Fig. 4*A* demonstrates the equal expression of PFKFB3 from FLAG-tagged constructs as determined by Western blot analysis. Remarkably, the WT PFKFB3-transfected cells proliferated at a significantly higher rate compared with cells transfected with empty vector ($9.8 \pm 0.84 \times 10^5$ cells *versus* $6.46 \pm 0.48 \times 10^5$ cells, $p < 0.001$), whereas expression of the K472A/K473A or R75A/R76A mutants had no effect on cell proliferation (Fig. 4*B*). A nearly identical result was obtained with the [^{3}H]thymidine incorporation assay and similar results were obtained in HCT116 cells (Fig. 4*C*, data not shown). The observation that the pro-proliferative effects of WT PFKFB3 were completely abrogated by mutating either the active site or nuclear localization residues of PFKFB3, demonstrates a requirement for nuclear delivery of Fru-2,6-BP.

Ectopic Expression of WT PFKFB3 Increases the Expression of Key Cell Cycle Proteins—A pathway-focused antibody array was conducted to identify cell cycle proteins that may be involved in mediating the effect of WT PFKFB3 on proliferation. HeLa cells were transiently transfected with either WT PFKFB3 or empty vector as control, and 36 h later, cells were collected and lysed. Analysis of the array results revealed induction in protein expression of several key cell cycle proteins by PFKFB3 including cyclin-dependent kinase-1 (Cdk1; Cdc2), Cdc25C, and cyclin D3 (Fig. 5, *A* and *B*). We next confirmed these results using Western blot analyses and found that protein expression levels of Cdk1, Cdc25C, and cyclin D3 were markedly up-regulated by ectopic expression of WT PFKFB3 (Fig. 5, *C* and *D*). Importantly, mutation of either the nuclear localization or active site residues of PFKFB3 attenuated the increased expression of each of the three cell cycle regulators (Fig. 5*C*). We also performed RT-PCR analysis and found no change in mRNA levels of each of these targets, suggesting that their regulation by PFKFB3 is post-transcriptional (data not shown).

Ectopic Expression of WT PFKFB3 Increases Phosphorylation of the Cell Cycle Inhibitor p27 at Threonine 187 and Decreases Total p27 Protein Levels—p27 (p27^{Kip1}; Kip1) is a member of the Kip/Cip family of proteins and a universal inhibitor of cyclin-dependent kinases (Cdk) and the cell cycle (29, 30). p27 protein expression negatively correlates with cellular proliferation and Cdk activity, as p27 is also a direct substrate for phosphorylation at threonine 187 (Thr-187) by two major cyclin-Cdk com-
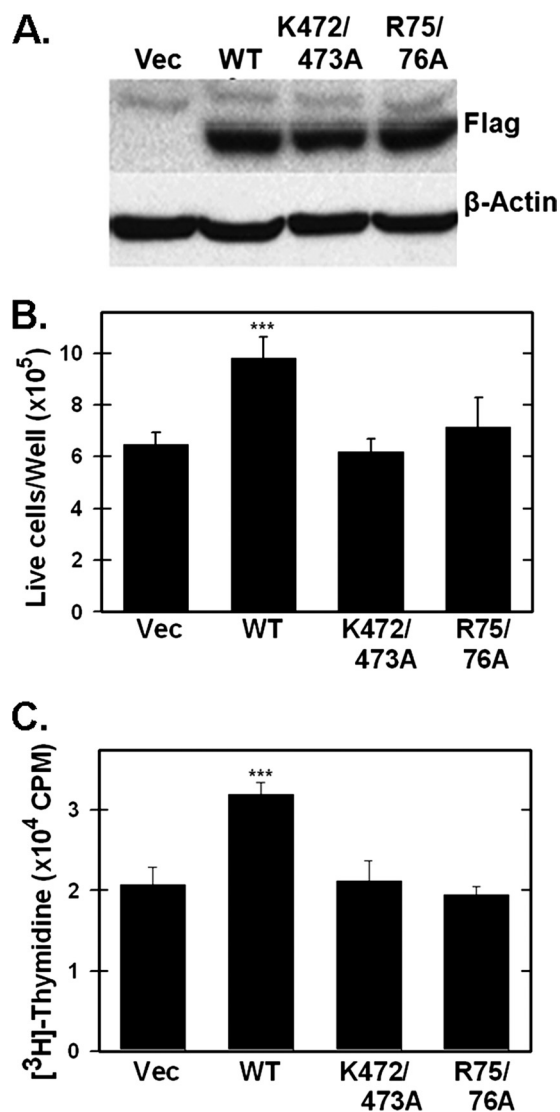

FIGURE 4. **Ectopic expression of a kinase-active PFKFB3 in the nucleus stimulates cell proliferation.** HeLa cells were transfected with wild type (*WT*), cytoplasmic (*K472/473A*), kinase-inactive (*R75/76A*) FLAG-tagged PFKFB3 constructs, or with empty vector (*Vec*) as control and 48 h post-transfection (*A*), Western blot was performed to confirm the equal expression of PFKFB3 from FLAG-tagged constructs; *B*, the trypan blue-excluding live cells were counted; and *C*, [^{3}H]thymidine incorporation into DNA was assessed. *Error bars* indicate mean ± S.D. of three independent experiments. *Asterisks* indicate $p < 0.001$.

plexes (namely cyclin B1/Cdk1 and cyclin A or E/Cdk2), the most potent determinant of p27 protein instability. We therefore analyzed p27 protein levels and phosphorylation state by Western blot and found that WT PFKFB3 decreased p27 protein levels and increased the relative phosphorylation of the p27 protein at Thr-187 compared with vector, K472A/K473A, and R75A/R76A samples (Fig. 5, *E* and *F*). These data suggest that Fru-2,6-BP production in the nucleus induces p27 phosphorylation at Thr-187, possibly leading to its ubiquitination and subsequent degradation by the proteasome (31, 32).

It has been well established that the p27 protein level oscillates during transition between cell cycle phases (33, 34) and it has been found in several cell lines that the p27 protein is degraded in G$_1$ and G$_1$/S phases (35–41). We sought to determine whether Fru-2,6-BP levels inversely correlate with p27
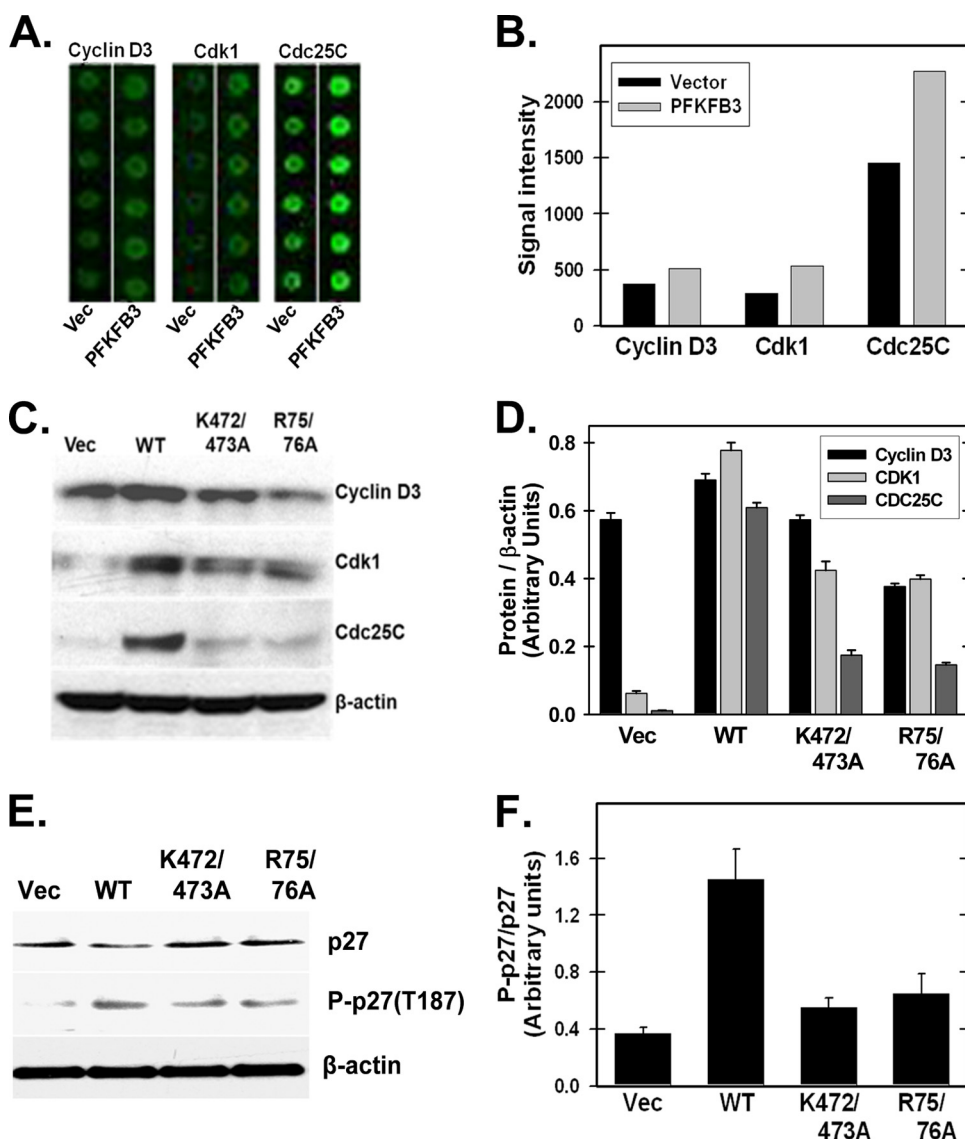
FIGURE 5. **Ectopic expression of a kinase-active, nuclear PFKFB3 regulates the expression of key cell cycle proteins and phosphorylation of the cell cycle inhibitor p27.** *A*, HeLa cells were transfected with either a construct carrying PFKFB3, or empty vector as control, and 36 h later, cells were lysed and subjected to antibody array. Portions of the array illustrating the differential expression of cyclin D3, Cdk1, and Cdc25 proteins between vector control (*Vec*) and PFKFB3-expressing (*PFKFB3*) cells are shown. Each panel contains six replicates of a specific antibody-protein reaction. *B*, densitometric quantitation of the average signal for each protein between samples. *C* and *D*, HeLa cells were transfected with WT, cytoplasmic (*K472/473A*), kinase-inactive (*R75/76A*) FLAG-tagged PFKFB3 constructs, or with empty vector (*Vec*) as control and 36 h post-transfection, cells were lysed and subjected to Western blot analysis using antibodies specific for cyclin D3, Cdk1, Cdc25C, and β-actin (*C*), and densitometric quantitation of the Western blot (*D*). *E* and *F*, HeLa cells were transfected as above and Western blot analysis was used to detect total and phosphorylated (threonine 187)-p27 levels (*E*) and densitometric quantitation of the Western blot (*F*). *Error bars* indicate mean ± S.D. of two experiments.

protein levels given that ectopic expression of PFKFB3 decreases p27. To this end, we synchronized HeLa cells at the G_1/S boundary using double-thymidine block and analyzed p27, PFKFB3, and Fru-2,6-BP levels in fractions enriched in S, G_2/M, and G_1 phases and found that the expression of the p27 protein was highest and the total cell steady-state concentration of Fru-2,6-BP was lowest in G_2/M phase (supplemental Fig. S3).

Fru-2,6-BP Increases p27 Phosphorylation at Thr-187 in Total HeLa Cell Lysates—Our findings suggest that Fru-2,6-BP synthesis in the nucleus is required for the effect on p27 protein

levels and its phosphorylation. To rule out the possibility that p27 phosphorylation by PFKFB3 is an indirect effect (*e.g.* secondary changes in gene expression) and to determine whether Fru-2,6-BP may directly regulate this event, we assessed p27 phosphorylation in total HeLa lysates incubated with bacterially expressed (*i.e.* unphosphorylated) GST-tagged p27, with or without addition of Fru-2,6-BP. Lysates were incubated for 25 min at room temperature and GST-p27 was pulled-down using glutathione beads followed by Western blot. As illustrated in Fig. 6, compared with control (no lysate), incubation of the unphosphorylated p27 in total lysates caused a detectable phosphorylation at Thr-187 as determined by Western blot and this was substantially enhanced by the addition of Fru-2,6-BP.

DISCUSSION

Although the PFKFB family of enzymes is not directly involved in the formation of a glycolytic intermediate, their product, Fru-2,6-BP, is considered to be essential in maintaining the high glycolytic flux as it is a potent allosteric activator of the rate-limiting enzyme of glycolysis, PFK-1 (7, 8, 20, 42, 43). PFKFB3 is distinguished from other enzymes by its high kinase to bisphosphatase ratio and has been demonstrated to be up-regulated in transformed cells and tumors (8, 11). Therefore, it is widely held that PFKFB3 is essential for the increased glycolytic flux in transformed cells.

In the recently solved crystal structure of PFKFB3, the carboxyl terminus appears flexible and disordered (28, 42), suggesting a discrete function. We have found that the dominantly expressed splice variant of PFKFB3 localizes to the nucleus and that the carboxyl terminus is required for this localization. A study performed by Telang *et al.* (44) demonstrated that the intracellular concentration of Fru-2,6-BP in murine fibroblasts isolated from PFKFB3-haploinsufficient mice was lower than their wild type counterparts, but that there was no significant difference in glycolysis. Moreover, our observations that cells express more than one PFKFB enzyme concomitantly and, among them, PFKFB2 and PFKFB4 localize to the cytoplasm where glycolysis takes place challenges the notion that PFKFB3 is a key activator

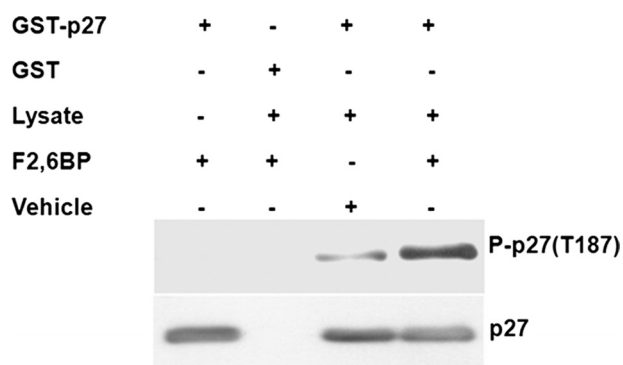| GST-p27	+	-	+	+
GST	-	+	-	-
Lysate	-	+	+	+
F2,6BP	+	+	-	+
Vehicle	-	-	+	-

FIGURE 6. **Fru-2,6-BP increases the phosphorylation of p27 at the Cdk-specific Thr-187 site in HeLa cell lysates.** Exponentially growing HeLa cells were lysed, and bacterially expressed GST-p27 (4 μg) and Fru-2,6-BP (200 μM) were added. p27 was pulled down and subjected to Western blot using total and Thr-187-phosphorylated p27 antibodies. Densitometric quantitation of the average signal for Thr-187-phosphorylated p27 relative to total p27 was conducted on protein preparations from three separate experiments.

of glycolysis and suggests a heretofore unidentified function for PFKFB3. Consistent with this hypothesis, we demonstrated in this study that ectopic expression of PFKFB3 in the nucleus did not have a significant effect on total cell glycolysis but rather stimulated cellular proliferation. We should emphasize, however, that our findings should not be interpreted as an argument against the well established role for Fru-2,6-BP in the regulation of glycolysis (43, 45–50). We suspect that Fru-2,6-BP may be primarily involved in the regulation of glycolysis in normal non-proliferating cells such as neurons and hepatocytes, whereas, in transformed cells, Fru-2,6-BP may serve a dual function in glycolysis and cell cycle regulation.

The observation that a large increase in Fru-2,6-BP production in cells by the ectopic expression of the wild type (*i.e.* nuclear) PFKFB3 did not stimulate the glycolytic flux, whereas the cytoplasmic mutant of PFKFB3 was able to enhance the glycolytic rate suggests the possibility for subcellular compartmentalization of Fru-2,6-BP. Concentration differences for small molecules such as calcium ions between subcellular compartments have previously been reported (51). Depending upon stimuli, there may exist a concentration gradient for Fru-2,6-BP between the cytoplasm and nucleus, so that a relative increase of Fru-2,6-BP in the cytoplasm may be necessary to activate glycolysis, whereas a relative increase in the nucleus may signal cells to more efficiently use glucose to proliferate (*i.e.* via the pentose phosphate pathway and oxidative phosphorylation in the mitochondria instead of anaerobic glycolysis). In line with this hypothesis, a study conducted by Vizan *et al.* (52) indicated that a specific K-Ras mutant (K12) markedly activated glycolysis, with only a minor effect on proliferation, whereas, another mutant (K13) forced cells to use the pentose phosphate pathway and proliferate, without affecting glycolysis significantly. This study suggested that glycolytic activity may not always correlate with proliferation.

Regulation of the cell cycle is complex and requires the coordinated actions of many key cell cycle proteins to overcome rate-limiting steps such as the G_1/S restriction point (30). Transformed cells such as HeLa and HCT116 are rapidly dividing cells and the observation that the ectopic expression of a protein can further enhance their proliferation is striking. For

nutrient signals to regulate cellular proliferation, these signals must be connected to the cell cycle machinery (53–59). Cyclin-dependent kinases promote cell cycle progression by phosphorylating substrates that control DNA replication, transcription, and mitosis (60–63). Our observation that PFKFB3 and Fru-2,6-BP can directly activate Cdk to phosphorylate p27 in HeLa lysates indicates that Fru-2,6-BP may have direct allosteric effects on Cdk1 or Cdk2 activity and future studies will be directed at this question.

Post-translational regulation of Cdk activity and the protein levels of p27 as well as cyclin D3 and Cdc25C by Fru-2,6-BP presents a number of interesting implications. First, several small metabolites generated in the cell serve as secondary messengers (*e.g.* cAMP). Our study suggests that the presence of Fru-2,6-BP in the nucleus may be required for cells to undergo cell division, which, without sufficient energy and macromolecule synthesis, can be catastrophic for cells. It can be speculated that, as a "by-product" of glucose metabolism, the concentration of Fru-2,6-BP in the nucleus reflects the rate of glucose uptake, and therefore, the presence of Fru-2,6-BP in the nucleus may be instrumental in coupling glucose uptake with cell proliferation. However, it is important to note that a total increase in glucose uptake and Fru-2,6-BP synthesis may not be sufficient for cells to increase proliferative capacity. Rather, Fru-2,6-BP synthesis *in the nucleus* may be essential to this process. Second, the high expression of PFKFB3 observed in tumors may be critical for their unrestricted growth, as Fru-2,6-BP may cause deregulation of Cdks, causing them to be less sensitive to normal inhibitory effectors. For example, Cdk1 requires cyclin B1 and cyclin A as partners for maximal activity, however, the expression of these cyclins is absent or minimal in the G_1 phase (64, 65), when Cdk1 is considered inactive. However, activation of Cdk1 by Fru-2,6-BP may result in early inactivation of p27 and Rb proteins, resulting in deregulation and shortening of the G_1 phase, which is a common feature of transformed cells (66–70). Taken together, these observations demonstrate an unexpected role for PFKFB3 in nuclear signaling and indicate that Fru-2,6-BP may couple the activation of glucose metabolism with cell proliferation. Furthermore, these studies should enable an improved understanding of the regulatory interplay between metabolism and cellular events associated with embryogenesis, development, and cancer.

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