Pyruvate kinase isoform M2 (PKM2) is an enzyme-catalyzing conversion of phosphoenolpyruvate to pyruvate in the glycolysis pathway. It was demonstrated that PKM2 interacts with tyrosine phosphopeptide, and the interaction with the tyrosine phosphopeptide affects the pyruvate kinase activity of PKM2. Our experiments suggest that PKM2 also interacts with tyrosine-phosphorylated proteins at the fructose 1,6-bisphosphate-binding site, converting the tetrameric PKM2 to a dimer. On the other hand, growth stimulations also lead to PKM2 phosphorylation, which consequently regulates the conversion of protein kinase and pyruvate kinase activities of PKM2 by different mechanisms. On the one hand, growth signals induce protein tyrosine phosphorylations. The tyrosine-phosphorylated protein(s) regulates the conversion of pyruvate kinase and protein kinase of PKM2 by directly interacting with PKM2. Binding of the tyrosyl-phosphorylated proteins at the fructose 1,6-bisphosphate-binding site converts the tetrameric PKM2 to a dimer. On the other hand, growth stimulations also lead to PKM2 phosphorylation, which consequently regulates the conversion of protein kinase and pyruvate kinase activities. Growth factor stimulations increase the dimer/tetramer PKM2 ratio and activate the protein kinase activity of PKM2. Our study suggests that the conversion between the pyruvate kinase and protein kinase activities of PKM2 may be an important mechanism mediating the effects of growth signals in promoting cell proliferation.

Pyruvate kinase isoenzyme M2 (PKM2) plays a very important role in controlling the flow of metabolites derived from glucose to either the biosynthesis pathway or metabolism pathways for energy production (1, 2). The glycolysis catalytically active PKM2 exists as a tetramer and associates with a glycolytic enzyme complex in normal cells (3–5). In tumor cells, PKM2 exists as a mixture of dimers and tetramers. It appears that the dimer PKM2 is catalytically inactive for conversion of phosphoenolpyruvate (PEP) to pyruvate (6, 7). It is believed that the inactive PKM2 actually provides growth advantage for tumor progression as it helps to channel the carbon source from glycolytic intermediates to biosynthesis, especially syntheses of nucleic acids, lipids, and proteins, to meet the demands for tumor cell proliferation. A number of recent studies suggest that growth signals regulate both localization and cellular function/activity of PKM2, especially its nonmetabolic functions. It is shown that PKM2 can bind to tyrosine phosphopeptide or become phosphorylated under growth stimulations. These interactions and modifications lead to reduction in pyruvate kinase activity of PKM2 (8, 9). Under epidermal growth factor (EGF) stimulation, PKM2 may translocate to the nucleus and co-activates the nuclear β-catenin to up-regulate expression of several key growth-related genes (10). PKM2 can also act as a Hif-1α co-activator in a feedback loop in response to hypoxia condition to re-program cancer cell metabolism during cancer growth (11). We previously demonstrated that PKM2 is also a protein kinase, and the protein kinase activity of PKM2 is critically important for cell proliferation (12). The protein kinase activity of PKM2 was also recently verified by a study showing that PKM2 directly phosphorylate histone H3 at Thr-11 in response to EGF stimulation (13). An open question is how the conversion between the protein kinase active dimer and pyruvate kinase active tetramer is regulated under growth conditions. We provide evidence here to show that growth signals promote PKM2 tetramer to dimer conversion by direct phosphorylation of the protein or interaction with tyrosine phosphoproteins in response to different growth stimulations. Our data also demonstrate that growth signals reciprocally regulate the protein kinase and pyruvate kinase activities by control of the dimer to tetramer status of PKM2 in cells.

**MATERIALS AND METHODS**

**Reagents, Cell Lines, and Antibodies**—The keyhole limpet hemocyanin-conjugated peptide spanning amino acids 399–
Tyrosine Phosphoprotein Regulates PKM2 Activities

413 of PKM2 (IYHQLFEELRLRAP) was synthesized by Global Peptide Services. PKM2 polyclonal antibody and anti-HA antibody were raised in the animal facility at Georgia State University. The peptides, D-PhosPepY593, PepY593, and Phos-PepY567, were synthesized by AnaSpec. Antibodies against Stat3, Stat3(Tyr(P)-705), anti-phosphorytrosine antibodies, Tyr(P)-100 and Tyr(P)-20, HA tag, GAPDH, Lamin A/C, and β-actin were purchased from Cell Signaling, Abnova, Santa Cruz Biotechnology, Abcam, and AnaSpec. Recombinant GST-Stat3 was purchased from Abcam. Cell lines SW480, Ba/F3, and T98G were purchased from the ATCC and cultured by following the vendor’s instructions.

Plasmids Construction—Human full-length cDNA of PKM2/PKM1 was purchased from OriGene Technologies. The cDNA of PKM2/PKM1 was subcloned into bacteria expression vector pET30α(+) as well as mammalian expression vector pHM6. The PKM2 or its mutants were fused at the C terminus of the HA protein tag in pHM6 mammalian expression vector. Site-directed mutagenesis were performed using QuikChange® multiple site-directed mutagenesis kit (Stratagene). All the DNA clones and mutations were verified by auto-DNA sequencing at Georgia State University. The HA-tagged full-length p68 (wild type, Y593F mutant) expression plasmids were constructed in pHM6 vectors as indicated in the previous papers from our laboratory (14). Immunoprecipitation, immunoblot analyses, recombinant PKM1/PKM2 expression and purification, peptide pull-down, size exclusion chromatography, in vitro phosphorylations, and pyruvate kinase assays followed the procedures similar to those described in our previous reports (12).

PKM2 and Peptide Interaction—Interactions between the rPKM2 and its derived mutants were analyzed by monitoring the changes of tryptophan fluorescence of the recombinant protein PKM2 or the mutants (λex = 285 nm and λem = 310–410 nm). The binding analyses were carried out at a protein concentration of 4 μM in the reaction buffer (50 mM Tris, pH 7.5, 100 mM KCl). Different peptides (100 μM) were titrated into the protein solution. All spectra were recorded at ambient temperature using a PTI spectrofluorimeter with a 1-cm path length cuvette. FBP was added to the protein solution at a final concentration of 2 mM.

RESULTS

Christofk and co-workers (9, 15) reported that PKM2 interacts with the phosphotyrosine peptide, and the interaction plays an important role in promoting cell proliferation, suggesting a possible pathway that growth signals promote proliferation via protein tyrosine phosphorylation and subsequent action on PKM2. Nevertheless, an actual tyrosine-phosphorylated cellular protein that interacts with PKM2 was not identified and demonstrated. We previously showed that p68 RNA helicase is phosphorylated at tyrosine residues in cancer cells, and tyrosine phosphorylation of p68 correlates with cancer progression (16). Phosphorylation of p68 at Tyr-593 promotes epithelial-mesenchymal transition (14), whereas phosphorylation of p68 at Tyr-593 and Tyr-595 mediated resistance to apoptotic induction (17). In an effort to probe the interacting protein with the tyrosyl-phosphorylated p68, we used a 14-amino acid segment that includes the Tyr-593 or Tyr-593/595 phosphorylation site (amino acids 587–600). The peptides either carried no phosphorylation (PepY593), Tyr-593 phosphorylation (PhosPepY593), or Tyr-593/595 double phosphorylations (D-PhosPepY593). We also used another control peptide that spans different regions of p68 (amino acids 561–574) with Tyr-567 phosphorylation (PhosPepY567). The peptides were conjugated to agarose beads. We used a glioma cell line T98G here, as we observed previously that p68 had strongest Tyr-593 and Tyr-593/595 phosphorylations in T98G cells (17). Precipitation experiments with nuclear extracts of T98G cells demonstrated that the D-PhosPepY593 pulled down a nuclear protein that migrated at ~60 kDa. The interacting protein was identified by matrix-assisted laser desorption ionization (MALDI) (TOF/TOF) as PKM2 (Fig. 1A). The p68 and PKM2 interaction was first verified by a peptide pulldown with rPKM2 and the pull-down with nuclear extracts of T98G cells using the rPKM2 as bait (Fig. 1, B and C). We also verified the interaction by co-immunoprecipitation with nuclear extracts of T98G cells (Fig. 1, D and E). To test whether the PKM2 and p68 interaction is p68 phosphorylation-dependent, we carried out co-immunoprecipitation experiment with the nuclear extracts of T98G cells in which HA-p68, WT, or Y593F (a p68 mutant that cannot be phosphorylated) was expressed. It was clear that WT HA-p68 co-precipitated with PKM2, although the Y593F mutant did not (Fig. 1D), suggesting that p68 tyrosine phosphorylation is required for PKM2 and p68 interaction.

Phosphorylation of p68 at tyrosine residues correlates with cancer cell proliferation, and growth stimulations induce tyrosine phosphorylation of p68 at tyrosine residues (16, 17). We speculated whether the interaction of PKM2 with the phosphorylated p68 might be a typical example of cellular tyrosine phosphoproteins that interact with PKM2 and play a role in the regulation of PKM2’s protein kinase and pyruvate kinase activities. To test this speculation, we employed three phosphorylated/unphosphorylated peptides, the D-PhosPepY593, the PhosPepY567, and the PepY593. We first examined the interaction of the selected peptides with the rPKM2 by monitoring the Trp fluorescence changes of the rPKM2. It was clear that the D-PhosPepY593 interacted with the rPKM2 with a Kd of around 30 μM, whereas the PhosPepY567 and the PepY593 did not (Fig. 2, A–C). It was suggested that the tyrosine phosphopeptide interacts with PKM2 at the FBP-binding site (9). We therefore examined whether the D-PhosPepY593 interacts with the rPKM2 at the FBP-binding site by testing the interaction in the presence of excessive amounts of FBP. It was clear that FBP competed with the D-PhosPepY593 (Fig. 2, A–C). The competition of FBP with p68 for binding to PKM2 was also revealed by co-immunoprecipitation experiments showing that co-precipitation of p68 with HA-PKM2 in T98G cell extracts decreased by supplementing the extracts with different concentrations of FBP (Fig. 3A). The similar co-immunoprecipitation experiment was also carried out with the rPKM2 and the HA-p68 immunopurified from T98G cell extracts in the presence of various concentrations of FBP. High concentration of FBP decreased the HA-p68 and the rPKM2 interaction (Fig. 3B). It is reported that Trp-515 to Gly-520 located at the activating loop of the C terminus of PKM2 is critical for the FBP binding (3). Thus, we created a truncation mutant with deletion of Trp-515 to Gly-531. The...
interaction of the phospho-p68 with the PKM2 truncation was examined by the co-immunoprecipitation procedure. It was evident that the deletion of the FBP-binding activating loop abolished the interaction between p68 and PKM2 (Fig. 3C). Meanwhile, a mutation (K270M) that affects the glycolytic substrate binding (3) did not affect the interaction between p68 and PKM2 (Fig. 3C). These results are consistent with the observations by Christofk and co-workers (9), indicating that the phosphopeptides D-PhosPepY593 and phospho-p68 interact with PKM2 at the FBP-binding site. We next examined the effects of binding the phosphopeptides on the tetramer and dimer formation. We used size exclusion chromatography method to analyze the molecular sizes of the rPKM2 in the presence or absence of the three tyrosine phospho/unphosphopeptides and compared them with the chromatography profile of molecular size standards. It was evident that there was substantially more dimeric PKM2 in the presence of D-PhosPepY593, whereas the tetramer and dimer ratio of the rPKM2 was not affected in the

**FIGURE 1. Tyrosine-phosphorylated p68 interacts with PKM2.** A, left panel, GelCode staining SDS-PAGE analyses of proteins pulled down from nuclear extracts of T98G cells by different peptides coupled to agarose beads. The peptides used are as follows: PepY593, PhosPepY593, D-PhosPepY593, and PhosPepY567. The arrow indicates the protein band that was cut out and subjected to trypsin digestion followed by MALDI-TOF/TOF analyses. M is molecular weight markers. Right panel, amino acid sequence of PKM2. The boldface and underlined sequences indicate the peptide fragments identified by MALDI-TOF/TOF. B, interaction of the peptides, the PepY593 and D-PhosPepY593, with rPKM2. The recombinant PKM2 pulled down (PD) by the peptides was examined by immunoblot (IB) using antibody against PKM2 (IB:PKM2). Pulldown by PKM2 is a control without peptide pulldown. C, pulldown (PD) of p68 from nuclear extracts (NE) of T98G cells by rPKM2 was examined by immunoblot using antibody p68-RGG (IB:p68). The tyrosine phosphorylation of p68 was indicated by strip-blot of the pulldown using antibody against phosphotyrosine (IB:PY20). D, co-immunoprecipitations of exogenously expressed HA-p68s (WT and Y593F, Vec is vector alone) with endogenous PKM2 from nuclear extracts of T98G cells were examined by immunoblot with anti-HA (IP:PKM2, IB:HA). IBs of the nuclear extracts using anti-HA indicate the expression of HA-p68 (IB:HA). IBs of histone 2A (IB:H2A) are the loading controls. E, top panel, co-immunoprecipitations of p68 with endogenous PKM2 from nuclear and cytoplasmic (Cyto) extracts of T98G cells were examined by immunoblot with antibody p68-RGG (IP:PKM2, IB:p68). Bottom panel, co-immunoprecipitations of p68 with exogenously expressed HA-PKM2 from nuclear and cytoplasmic extracts of T98G cells were examined by immunoblot with antibody p68-RGG (IP:HA, IB:p68). IBs of GAPDH (IB:GAPDH) and lamin A/C (IB:Lamin A/C) are the loading controls. Beads in B and C are the control pulldown using agarose beads alone.
The presence of the PhosPepY567 and the nonphosphopeptide PepY593 (Fig. 3D). The experiments apparently suggested that binding the tyrosyl phosphopeptide converts tetramer PKM2 to a dimeric form.

We then asked whether binding of the tyrosyl phosphoprotein/peptide would activate the protein kinase activity and inactive pyruvate kinase activity of PKM2. To this end, the in vitro phosphorylation reactions using GST-Stat3 as substrate and PEP as phosphate donor were carried out with the rPKM2 in the presence of the different peptides. Examination of phosphorylation of Stat3 by the immunoblot using the anti-phospho-Stat3 antibody indicated that phosphorylation of Stat3 by the rPKM2 was dramatically increased in the presence of D-PhosPepY593 (Fig. 4A). The phosphorylations of Stat3 by the rPKM2 were not affected in the presence of the PepY593. The phosphorylation of Stat3 was also not affected in the presence of peptide PhosPepY567 that did not bind to PKM2 (Fig. 4A).

As a control, the peptide D-PhosPepY593 did not affect the phosphorylation of GST-Stat3 by R399E, and the peptide alone did not lead to Stat3 phosphorylation (Fig. 4B). We next tested
whether indeed the presence of phospho-p68 would activate the protein kinase activity of PKM2. To this end, we immuno-purified HA-p68 from nuclear extracts of T98G cells. Phosphorylation of GST-Stat3 by the rPKM2 (10 μg/ml) and the R399E (10 μg/ml) (A) or by R399E (10 μg/ml) (B) in the presence of different peptides (3 μM) D-PhosPepY593, PepY593, and PhosPepY567 was revealed by immunoblot assays using antibody against Tyr-705-phosphorylated Stat3 (IB:Y-Stat3). C, phosphorylation of GST-Stat3 by the rPKM2 (10 μg/ml) or the rPKM1 (10 μg/ml) in the presence of HA-p68 that was immunopurified from nuclear extracts of T98G cells and 5 mM of PEP was revealed by immunoblot assays using antibody against Tyr-705-phosphorylated Stat3 (IB:Y-Stat3). In some phosphorylation reactions, 5 mM FBP or 5 mM ADP was added to the reaction. Immunoblots of Stat3 (IB:Stat3), HA-p68 (IB:HA), and PKM2 (IB:PKM2) indicate the amounts of GST-Stat3, HA-p68, and the rPKM2 used in each phosphorylation reaction. D, phosphorylation of Stat3 by immunopurified HA-PKM2 from nuclear extracts (HAPK) of T98G cells in the presence of ATP or PEP was analyzed by immunoblot using the antibody against the Tyr-705-phosphorylated Stat3 (IB:Y-Stat3). p68 was knocked down by duplex RNAi. Immunoblot of Stat3 (IB:Stat3) shows the amounts of Stat3 that were immunoprecipitated from the nuclear extracts. Immunoblot of HA (IB:HA) indicates the expression levels of HA-PKM2. Ponceau S stain of IgG heavy chain (IgG:HC) indicates the amounts of antibody used in each PKM2 purification from nuclear extracts. E, pyruvate kinase activity of the rPKM2 (5 μg/ml) in the presence of different peptides (2 μM) D-PhosPepY593, PepY593, and PhosPepY567 or buffer was analyzed by the method described by Christofk et al. (9). The pyruvate kinase activity of the rPKM2 was expressed as relative pyruvate kinase activity by defining the activity in the presence of buffer as 100. The error bars represent the standard deviations of four measurements.

PKM2 is a glycolysis enzyme. It is believed that the tetramer form of the protein is catalytically active in catalyzing the conversion of PEP to pyruvate in the glycolysis. Because the binding of the tyrosine phosphoprotein promoted the conversion from tetramer to dimer, we reasoned that binding the tyrosine phosphoprotein/peptide to PKM2 should result in a protein that is less active in catalyzing the conversion of PEP to pyruvate. We used the method similar to that described by Christofk and co-workers (9) to monitor the pyruvate kinase activity of the rPKM2 in the presence of the tyrosine phosphopeptides. The rPKM2 catalytic activity was dramatically reduced in the presence of D-PhosPepY593, whereas the activity was not affected in the presence of the peptides PhosPepY567 and PepY593 (Fig. 4E), which indicate that binding of PKM2 to the tyrosine phosphoproteins converted the protein to a dimer and consequently reduced the glycolytic enzyme activity. The notion was further supported by the experiments that the pyruvate kinase activity of the R399E was dramatically decreased compared with that of recombinant wild type (12). In addition, examination of p68 and Stat3 phosphorylation levels in different cancer cells revealed a correlation (Fig. 5, A and B), suggesting a potential
FIGURE 5. A, top panels, phosphorylation of nuclear Stat3 was analyzed by immunoblot using antibody against Tyr-705-phosphorylated Stat3 (IB:pY-Stat3) in the nuclear extracts prepared from different cells. Immunoblot of Stat3 (IB:Stat3) indicates the Stat3 levels in the nuclear extracts. Immunoblot of lamin (IB:Lamin A/C) is a loading control. Bottom panels, phosphorylation of p68 in nuclear extracts was analyzed by immunoblot of the immunoprecipitated p68 (IP:p68) using the antibody Tyr(P)-100 (IB:pY-100). Immunoblot of p68 (IB:p68) indicates the amounts of p68 that were immunoprecipitated from nuclear extracts (IB:p68). B, quantitation of phosphorylation levels of Stat3 (upper panel) and the immunoprecipitated p68 (bottom panel) in nuclear extracts of different cells. The phosphorylation levels of Stat3 and immunoprecipitated p68 were presented as relative phosphorylation levels (pY-Stat3/Stat3) and (pYp68/p68), respectively. The error bars are standard deviations from four independent experiments. C, interaction of GST-Stat3, the bacterially expressed rPKM2, and the HA-p68 immunopurified from T98G cells was analyzed by GST-pulldown using the glutathione beads. The GST-Stat3 (2 μg/ml), the rPKM2 (2 μg/ml), and the HA-p68 were incubated in 500 μl at room temperature. The GST-Stat3 was precipitated by the GST beads. Co-precipitations of the HA-p68 and the rPKM2 from the mixture were analyzed by immunoblot using antibodies against p68 (IB:p68) and PKM2 (IB:PKM2). The strip blot using anti-phosphotyrosine indicates the tyrosine phosphorylation of the precipitated HA-p68. Immunoblot of Stat3 (IB:Stat3) indicates the amounts of GST-Stat3 that were precipitated by GST beads. D, co-immunoprecipitations of PKM2 and Stat3 with HA-p68 in the cellular extracts of T98G cells. The exogenously expressed HA-p68 was immunoprecipitated using anti-HA antibody. The amounts of p68 precipitated from the extracts were examined by immunoblot using antibody against p68. The co-precipitation of PKM2 and Stat3 was probed by immunoblot using antibodies against PKM2 (IB:PKM2) and against Stat3 (IB:Stat3). IgG HC in A and D are the Ponceau S stain of antibody heavy chain, representing the amounts of antibody used in immunoprecipitation of HA-p68 from the extracts.
role of phospho-p68 in activating the PKM2 protein kinase activity in the phosphorylation of Stat3 in cancer cells.

Our experiments thus far demonstrate that tyrosine phospho-p68 interacts with PKM2 at the FBP site and strengthens the protein kinase activity of the protein. We therefore predict a three-way interaction among the phospho-p68, PKM2, and Stat3. To test this possible protein interaction, we carried out the GST pulldown using GST-Stat3, rPKM2, and HA-p68 that were immunopurified from T98G cell extracts. Immoblot demonstrated that both the rPKM2 and HA-p68 were co-purified with GST-Stat3 (Fig. 5C). The three-way interaction was also probed by co-immunoprecipitation experiments using anti-HA antibody. rPKM2 and GST-Stat3 were co-immunoprecipitated with HA-p68 that was immunopurified from cell extracts of T98G cells (Fig. 5D). These results provided additional support for the notion that interaction with tyrosyl phosphoprotein increased protein kinase activity of PKM2. The results also provide additional support for the notion that protein kinase substrate binds to the ADP-binding site of PKM2.

It is well documented that stimulation of cells by growth factors and cytokines results in activation of various protein-tyrosine kinases that subsequently phosphorylate many protein substrates (18). For instance, we previously demonstrated that platelet-derived growth factor (PDGF) or EGF stimulation led to tyrosine phosphorylation of p68 RNA helicase in SW480 cells (16, 19). Presently, our experiments demonstrated that binding to tyrosine phosphoprotein converted PKM2 from tetramer to dimer and therefore activated the protein kinase activity. We reasoned whether treatment of cells with growth factors would affect PKM2. Because T98G cells do not respond well to EGF treatment, we used a colon cancer cell line SW480 that is known to respond very well to EGF treatment. SW480 cells were treated with EGF. The same chromatography procedure used for detecting the PKM2 tetramer versus dimer in crude cell extracts was employed here to analyze the tetramer and dimer ratio of PKM2 in cells under the stimulation of EGF. It was clear that the levels of dimer PKM2 in growth factor-stimulated cells were significantly higher than that in the corresponding unstimulated cells (Fig. 6A). Immoblot analyses of PKM2 in nuclear and cytoplasmic extracts suggested that growth factor stimulation led to an increase in nuclear PKM2 levels (Fig. 6B). Analyses of protein tyrosine phosphorylation and p68 tyrosine phosphorylation revealed that there was a substantial increase in protein tyrosine phosphorylation and tyrosine phosphorylation of p68 at a tyrosine residue and an increase in p68 and PKM2 interaction, whereas the pyruvate kinase activity of the cell extracts prepared from the cells that were treated by the growth factor decreased (Fig. 6C). These experiments suggested that indeed there is a clear correlation between the levels of protein tyrosine phosphorylations and dimeric PKM2 in cells, which is inducible by growth factors and cytokines.

Hitosugi et al. (8) recently reported that PKM2 became phosphorylated at Tyr-105 upon fibroblast growth factor (FGF)
Tyrosine Phosphoprotein Regulates PKM2 Activities

FIGURE 7. A, phosphorylations of GST-Stat3 by PKM2 immunopurified from extracts of Ba/F3 cells using antibodies against PKM2 (αPK) or Tyr-105-phosphorylated PKM2 (αpY105) were analyzed by immunoblot using antibody against Tyr-705-phosphorylated Stat3 (IBpY-Stat3). IB:PKM2 indicates the amount of PKM2 that immunoprecipitated from the extracts. IgG HC is the IgG heavy chain indicating the amount of antibody used in the immunoprecipitation. B, phosphorylations of GST-Stat3 by the rPKM2 (10 μg/ml), rR399E (10 μg/ml), or rY105E (10 μg/ml) in the presence of 2 mM PEP were analyzed by immunoblot using antibody against Tyr-705-phosphorylated Stat3 (IBpY-Stat3). CBB is Coomassie Brilliant Blue stain of GST-Stat3 indicating amount of GST-Stat3 used in each phosphorylation reaction. C, in vitro phosphorylations of GST-Stat3 by recombinant Y105E and Y105F PKM2 mutants in the presence and absence of phosphorylated HA-p68 immunopurified from T98G cells were analyzed by immunoblot using antibody against Tyr-705 phosphorylated Stat3 (IBpY-Stat3). IB:HA indicates the amount of HA-p68 used in each phosphorylation reaction. IB:Stat3 indicates the amount of GST-Stat3 used in each phosphorylation reaction. D, hypothetical model that illustrates the functional role of growth signals in the regulation of protein kinase and pyruvate kinase activities of PKM2.

stimulation, and the phosphorylation promotes the PKM2 from tetramer to dimer transition. The phosphorylation also reduces the pyruvate kinase activity. We questioned whether the Tyr-105-phosphorylated PKM2 also increased its protein kinase activity. We immunoprecipitated the Tyr-105-phosphorylated PKM2 or total PKM2 using the antibodies against PKM2 or the Tyr-105-phosphorylated PKM2 from extracts of Ba/F3 cells that stably express FGF receptor (8). The precipitated PKM2 or the Tyr-105-phosphorylated PKM2 was then employed to carry out the same in vitro phosphorylation reaction using GST-Stat3 as substrate. Immunoblot analyses using the anti-Tyr(P)-705-Stat3 showed that the GST-Stat3 gained much stronger phosphorylation by the PKM2 immunopurified using the antibody against Tyr-105-phosphorylated PKM2 compared with that using antibody against PKM2 (Fig. 7A). To further confirm the protein kinase activity of Tyr-105-phosphorylated PKM2, we created a phosphomimic mutant Y105E. The in vitro phosphorylation reactions were carried out with the bacterially expressed wild type PKM2 and R399E and Y105E mutants. Immunoblots demonstrated that GST-Stat3 was phosphorylated by the recombinant Y105E (Fig. 7B). These results suggest that growth stimulation by FGF may also facilitate the activation of protein kinase activity of PKM2 by direct PKM2 post-translational modification.

We showed that the protein kinase activity and pyruvate kinase activity are regulated by interaction between tyrosine phosphoproteins and PKM2 and/or by phosphorylation of PKM2 under growth stimulation. We questioned whether these two regulatory mechanisms affect each other. To this end, we tested the in vitro phosphorylation of GST-Stat3 by PKM2 mutants Y105E and Y105F in the presence of tyrosine-phosphorylated p68. It is evident that phosphorylation of GST-Stat3 by recombinant Y105E is enhanced by the presence of the phospho-p68. Interestingly, phosphorylation of GST-Stat3 by Y105F is also enhanced by the presence of the phospho-p68 (Fig. 7C). The results indicate that regulations of protein kinase/pyruvate kinase activities of PKM2 by interacting with tyrosine phosphoproteins or by PKM2 itself phosphorylation are not dependent, reflecting that the conversion is subjected to regulation by multiple growth signals.

DISCUSSION

During tumor progression, growth signals stimulate the conversion of glycolytically active PKM2 to an inactive form, consequently regulating the glycolysis pathway to channel the carbon source from glucose for biosynthesis (15, 20–22). It is conceivable that tumor cells need to coordinate the metabolic alterations with expression of genes that are related to cell proliferation. The functions of PKM2 in regulating expression of genes fulfill the role of feedback signaling from metabolic alterations to gene regulation during tumor malignancy transformation (see model in Fig. 7D).

We show that protein kinase activity of PKM2 is associated with proliferation. Thus, it is conceivable that the growth signals must also regulate protein kinase activity of PKM2. Our studies suggest here that the conversion between the pyruvate kinase and protein kinase activities of PKM2 are regulated by multiple growth signals by different mechanisms as follows. 1) Tyrosine phosphorylations of various cellular proteins under growth stimulations facilitate tetramer to dimer conversion of PKM2 by direct interaction. This conversion leads to inactivation of pyruvate kinase and activation of protein kinase activities of PKM2. 2) Growth signals can also lead to direct phosphorylation of PKM2 (8). Phosphorylation of PKM2, e.g. at Tyr-105, results in converting the tetramer pyruvinate kinase active form to a dimer protein kinase active form. The reciprocal and reversible regulation of pyruvate kinase and protein kinase activities of PKM2 functions in altering cell metabolism and coordinating the metabolic adjustments with other cellular processes in response to growth stimulations. It is intriguing that interaction with FBP facilitates PKM2 to form a tetramer, and binding to tyrosine phosphoproteins promotes a dimer formation. Why these phosphomolecules have just opposite effects is an open question.

Acknowledgments—We are grateful for the comments from Natalie White, Christie Carter, and Yun Huang.
REFERENCES

1. Elbers, J. R., van Unnik, J. A., Rijken, G., van Oirschot, B. A., Roholl, P. J., Oosting, J., and Staal, G. E. (1991) Pyruvate kinase activity and isozyme composition in normal fibrous tissue and fibroblastic proliferations. Cancer 67, 2552–2559
2. Hacker, H. J., Steinberg, P., and Bannasch, P. (1998) Pyruvate kinase isoenzyme shift from L-type to M2-type is a late event in hepatocarcinogenesis induced in rats by a choline-deficient/DL-ethionine-supplemented diet. Carcinogenesis 19, 99–107
3. Dombrauckas, J. D., Santarsiero, B. D., and Mesecar, A. D. (2005) Structural basis for tumor pyruvate kinase M2 allosteric regulation and catalysis. Biochemistry 44, 9417–9429
4. Zwerschke, W., Mazurek, S., Massimi, P., Banks, L., Eigenbrodt, E., and Jansen-Dürr, P. (1999) Modulation of type M2 pyruvate kinase activity by the human papillomavirus type 16 E7 oncoprotein. Proc. Natl. Acad. Sci. U.S.A. 96, 1291–1296
5. Altenberg, B., and Greulich, K. O. (2004) Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. Genomics 84, 1014–1020
6. Mazurek, S., Boschek, C. B., Hugo, F., and Eigenbrodt, E. (2005) Pyruvate kinase type M2 and its role in tumor growth and spreading. Semin. Cancer Biol. 15, 300–308
7. Ashizawa, K., Willingham, M. C., Liang, C. M., and Cheng, S. Y. (1991) In vivo regulation of monomer-tetramer conversion of pyruvate kinase sub-type M2 by glucose is mediated via fructose 1,6-bisphosphate. J. Biol. Chem. 266, 16842–16846
8. Yang, W., Xia, Y., Sun, S. Y., Zhao, S., and Liu, Z. R. (2007) A double tyrosine phosphorylation of p68 RNA helicase confers resistance to TRAIL-induced apoptosis. Oncogene 26, 6082–6092
9. Hunter, T., and Cooper, J. A. (1985) Protein-tyrosine kinases. Annu. Rev. Biochem. 54, 897–930
10. Yang, W., Xia, Y., Ji, H., Zheng, Y., Liang, J., Huang, W., Gao, X., Aldape, K., and Lu, Z. (2011) Nuclear PKM2 regulates β-catenin transactivation upon EGFR activation. Nature 480, 118–122

Tyrosine Phosphoprotein Regulates PKM2 Activities

11. Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O’Meally, R., Cole, R. N., Pande, A., and Semenza, G. L. (2011) Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell 145, 732–744
12. Gao, X., Wang, H., Yang, J. J., Liu, X., and Liu, Z. R. (2012) Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. Mol. Cell 45, 598–609
13. Yang, W., Xia, Y., Hawke, D., Li, X., Liang, J., Xing, D., Aldape, K., Hunter, T., Alfred Yung, W. K., and Lu, Z. (2012) PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. Cell 150, 685–696
14. Yang, L., Lin, C., and Liu, Z. R. (2006) p68 RNA helicase mediates PDGF-induced epithelial mesenchymal transition by displacing Axin from β-catenin. Cell 127, 139–155
15. Christofk, H. R., Vander Heiden, M. G., Harris, M. H., Ramanathan, A., Gerszten, R. E., Wei, R., Fleming, M. D., Schreiber, S. L., and Cantley, L. C. (2008) The M2 splice isofrom of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452, 230–233
16. Yang, L., Lin, C., and Liu, Z. R. (2005) Phosphorylations of DEAD box p68 RNA helicase are associated with cancer development and cell proliferation. Mol. Cancer Res. 3, 355–363
17. Yang, L., Lin, C., Sun, S. Y., Zhao, S., and Liu, Z. R. (2007) A double tyrosine phosphorylation of p68 RNA helicase confers resistance to TRAIL-induced apoptosis. Oncogene 26, 6082–6092
18. Hunter, T., and Cooper, J. A. (1985) Protein-tyrosine kinases. Ann. Rev. Biochem. 54, 897–930
19. Yang, L., Lin, C., Zhao, S., Wang, H., and Liu, Z. R. (2007) Phosphorylation of p68 RNA helicase plays a role in platelet-derived growth factor-induced cell proliferation by up-regulating cyclin D1 and c-Myc expression. J. Biol. Chem. 282, 16811–16819
20. Deberardinis, R. J., Sayed, N., Ditsworth, D., and Thompson, C. B. (2008) Brick by brick: metabolism and tumor cell growth. Curr. Opin. Genet. Dev. 18, 54–61
21. Garber, K. (2006) Energy deregulation: licensing tumors to grow. Science 312, 1158–1159
22. Mazurek, S., and Eigenbrodt, E. (2003) The tumor metabolome. Anticancer Res. 23, 1149–1154