Dominant Negative Mutations Affect Oligomerization of Human Pyruvate Kinase M2 Isozyme and Promote Cellular Growth and Polyploidy*†

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This study was designed to understand the mechanism and functional implication of the two heterozygous mutations (H391Y and K422R) of human pyruvate kinase M2 isozyme (PKM2) observed earlier in a Bloom syndrome background. The co-expression of homotetrameric wild type and mutant PKM2 in the cellular milieu resulting in the interaction between the two at the monomer level was substantiated further by in vitro experiments. The cross-monomer interaction significantly altered the oligomeric state of PKM2 by favoring dimerization and heterotetramerization. In silico study provided an added support in showing that hetero-oligomerization was energetically favorable. The hetero-oligomeric populations of PKM2 showed altered activity and affinity, and their expression resulted in an increased growth rate of Escherichia coli as well as mammalian cells, along with an increased rate of polyploidy. These features are known to be essential to tumor progression. This study provides insight in understanding the modulated role of large oligomeric multifunctional proteins such as PKM2 by affecting cellular behavior, which is an essential observation to understand tumor sustenance and progression and to design therapeutic intervention in future.

A variety of genetic diseases and experimental situations within the heterozygous state depict an allelic relationship where the mutant recessive allele overrides the function of its normal (wild type) dominant allele. This condition, referred to as dominant negative, is usually observed in the case of oligomeric or multidomain proteins with a possibility of cross-monomer interaction. Such mutant proteins by acting as competitive inhibitors of the normal protein function could generate polymorphic forms in a single cell. A phenomenon observed in collagen, where dominant negative mutations cause the production of abnormal oligomers (1, 2), and in transcription factors like helix-loop-helix and leucine zippers, where mutant monomers sequestering the function of wild type in a dimer bound to DNA, leads to an altered gene expression (3–5). Dominant negative mutations are also reported to affect some multifunctional molecules like p53 with differential impact on cell physiology (6–10).

Pyruvate kinase (EC 2.7.1.40) catalyzes irreversibly the transphosphorylation from P-enolpyruvate to ADP-generating pyruvate and ATP in glycolysis (11, 12). Depending upon the differential metabolic requirements of the tissues, the enzyme is expressed in four different isoforms, L, R, M1, and M2 in vertebrates (13). PKM2 is a ubiquitous, prototype enzyme, present in all tissues during embryonic stage, and is gradually replaced by other isozymic forms in specific tissues, during development. It is necessary for cellular division irrespective of the type of tissue and reappears during cellular division and tumor formation (14–17). PKM2 is known to regulate its activity by switching between an active tetramer and inactive dimer form in a fructose 1,6-bisphosphate-dependent manner to shift the cellular metabolism accordingly during cell division. Hence, its role is implicated in cancer progression (14). Because any known aberration of PKM2 is not yet observed in more than one naturally occurring pathological condition, its role as a “metabolic modulator” has remained enigmatic.

This study deals with two missense mutations detected by us from a patient with BS, prone to various types of cancers at early age, and a BS cell line (18, 19). Both K422R and H391Y mutant proteins individually were shown to maintain tetrameric structure, despite mutations in the ISCD domain, while showing a differential loss of activity. One of the mutants, H391Y, had shown a significant structural rigidity, thermostability, stability over a range of pH, and a loss of typical allosteric behavior. We hypothesized how a multifunctional protein modulates its structure and regulates its function to adapt to stressful conditions like cancer to favor tumor promotion (20). Because the mutations observed were dominant negative in nature and were observed in vivo under heterozygous conditions, it was pertinent to understand the nature of interaction between wild type and mutant proteins under bi-allelic expression conditions. The cross-monomer interactions between wild type and mutant PKM2 shifted a tetramer ensemble to less active dimeric

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The abbreviations used are: PKM2, human pyruvate kinase M2 isozyme; PK-WT or PKWT, wild type PKM2; GST-PKWT, glutathione S-transferase-fused PKWT; His-PKWT, His-tagged PKWT; ISCD, inter-subunit contact domain; BS, Bloom syndrome; GFP, green fluorescent protein; RFP, red fluorescent protein; FACS, fluorescence-activated cell sorter; r.m.s.d., root mean square deviation.
and heterotetrameric forms, adding another dimension to the mechanism of alteration of PKM₂ activity, as a result of dominant negative mutation with its functional relevance.

**EXPERIMENTAL PROCEDURES**

**Clone Construction**—Wild type and mutant human PKM₂ cDNA were cloned in different vectors using unique restriction sites. Cloning in pGAD-C1 and pGBK-C1 vectors was performed for yeast two-hybrid assays. Cloning in pET28a(+), pGEX-4T1, and pRSET-B vectors was carried out for independent expression and co-expression of wild type and mutant proteins in bacterial cells and for GST pulldown assay. pDsRed-C1 and pEGFP vectors were used for co-localization experiments in HeLa cells. For simultaneous co-expression of wild type as well as mutant proteins in a mammalian cell, the cloning was also carried out in pBI-Tet vector with a bidirectional promoter. The restriction enzymes used for different vectors, primer sequences, and PCR conditions are provided in supplemental Table S1.

**Yeast Two-hybrid and GST Pulldown Assay**—pGBD-C1 vector containing PK-WT (wild type PKM₂) cDNA was co-transformed in the AH109 yeast strain with pGAD-C1 vector containing mutant (H391Y and K422R) PKM₂ cDNA, following the manufacturer’s protocol (Yeastmaker™ YT System-2, Clontech). The colonies were selected on Trp(−) and Leu(−) media and screened for positive interaction on triple dropout media containing PK-WT (wild type PKM₂) cDNA and grown on Trp(−)/H11002, Leu(−)/H11002, and His(−)/H11002 media containing 20 mM 3-amino-1,2,4-triazole (Sigma). The yeast cells were transformed with pGBK-C1 carrying PK-WT cDNA and grown on Trp(−), His(−) media to check the transactivation property of PKM₂. For standard GST pulldown assay, 5–5 μg of independently expressed and purified GST-fused PK-WT and His-tagged mutant proteins from Escherichia coli were mixed and incubated in binding buffer (21). The bound protein complex was later pulled out using glutathione-agarose beads (Sigma), washed, and loaded on 12% SDS-PAGE. A mixture of GST protein alone with His-tagged PK-WT and respective mutants was used as negative controls, whereas GST-PKWT with His-PKWT acted as a positive control. In addition, a “modified GST pulldown assay” was performed by co-expressing GST-fused PK-WT and His-tagged mutant proteins in BL21 DE3 E. coli strain, using two different expression vectors with respective antibiotics. Cell lysate was passed through a glutathione-agarose column and washed well, and the eluted complex was analyzed on 12% SDS-PAGE. Co-expression of GST alone (26 kDa) with His-tagged PKM₂ (−60 kDa) was taken as a negative control. Western blot was performed using anti-human PKM₂ monoclonal antibodies (Cell Signaling Technology).

**Gel Permeation, Glycerol Gradient, and Glutaraldehyde Cross-linking Assay**—To study the effect of co-expression of wild type and mutant PKM₂ on the oligomeric state of the enzyme, 5 mg of co-expressed protein (His-tagged wild type and His-tagged mutant PKM₂) was loaded in a Superdex 300 Superfine (Amersham Biosciences) gel permeation column, and the rest of the procedure adopted was the same as described earlier (20). However, to study the possibility of heterotetramerization, a GST-fused monomer of PK-WT (86 kDa) was co-expressed with His-tagged mutant protein (60 kDa) in E. coli to allow for the generation of heterotetramers of various sizes (ranging from 266–318 kDa) depending upon the stoichiometry of cross-monomer interaction with a possibility of independent wild type and mutant homotetramers (344 and 240 kDa, respectively). The E. coli cell lysate (500 μg of protein) with co-expressed wild type and mutant proteins was loaded at the top of a 15–33% glycerol gradient (using 50 mM Tris-Cl buffer, pH 8, containing 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 50,000 rpm for 16 h at 4 °C using an SW-55 Ti Beckman Coulter rotor. Because cancer cells are reported to overexpress a stable PKM₂ dimer besides tetramer, the lysate (500 μg of protein) from the HT1080 mammalian cell line was also loaded in a similar way. The co-expression of GST-PKWT and His-PKWT was taken as a positive control to assess peak position. Furthermore, to analyze the formation of hetero-oligomers in mammalian cells, HeLa cell lysate (500 μg of protein) with co-expressed GFP-fused PKWT (~86 kDa) and mutant protein (~60 kDa) was loaded on glycercol gradient. Fractions of 75 μL were taken from the gradient and assayed for PKM₂ activity, which was dependent upon the proportionate representation of homo- and heterotetramers formed. The activity (units/ml) was plotted against the sample number. For the glutaraldehyde cross-linking assay, co-expressed and purified GST-wild type and His mutant protein was incubated in 50 mM phosphate buffer, pH 8, with 0.1% glutaraldehyde in ice for 30 min and loaded on 6% SDS-PAGE for analyses.

**Protein Purification, Activity Assay, Enzyme Kinetics, and Bacterial Growth Curve**—Protein purification from E. coli, estimation, and activity assays were performed as described earlier (20). P-enolpyruvate saturation kinetics was performed by taking an equal amount of co-expressed wild type-wild type and each of the wild type-mutant PKM₂ proteins at various concentrations (0–5 mM) of P-enolpyruvate. The initial velocity data were fit to the Michaelis-Menten equation for calculating values of Kₘ and Vₘₐₓ as described earlier (20). Growth curve of BL21DE3 E. coli, co-expressing GST-fused PK-WT and His-tagged mutant proteins (from pGEX-4T1 and pET28a(+), respectively), was made by inducing the protein expression at 0 h and measuring A₅₆₀ of the culture at every 2-h interval. As pGEX-4T1 and pET28a(+) harbor different promoters (Tac and T7 respectively), to avoid bias in expression level of the two proteins, we cloned PK-WT cDNA in pRSET-B vector containing T7 promoter to match the efficiency of pET28a(+) expression. After optimizing the PK-WT expression under the T7 promoter in pRSET-B vector and confirming the equal expression of both proteins, both the vectors containing wild type and mutant cDNA under similar promoter were co-transfected, and the bacterial growth curve was studied.

**Mammalian Cell Culture, Transfection, FACS, and Confocal Study**—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal bovine serum (Biowest) with appropriate antibiotics, although HT1080-N1 cells were maintained as described (22). Mammalian cell lysate was prepared by incubating the cells in 50 mM Tris-Cl, pH 7.5, containing 1 mM EDTA, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethyalsulfonyl fluoride, 1 mM dithiothreitol, and protease inhibitor mixture in ice for 30 min. The lysate was centrifuged

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**Functional Implications of Human PKM₂ Mutations**
Functional Implications of Human PKM₂ Mutations

at high speed and supernatant collected. Transfections were carried out using Polyfect transfection reagent (Qiagen), using the manufacturer’s protocol. The protein expression of transfected clones was confirmed by activity assays after 48 h of transfection. Cell counting was also performed at the same time, using a hemocytometer. For flow cytometric (FACS) analysis, cells were harvested after 48 h of transfection, pelleted down, washed with phosphate-buffered saline and treated with 70% chilled ethanol. Cells were then incubated with RNase and propidium iodide (Sigma) at 37 °C and used for FACS (FACScalibur from BD Biosciences). The confocal study was carried out using HeLa cells, co-expressing GFP-tagged wild type and RFP-tagged mutant PKM₂ after 24 h of co-transfection of respective clones (pEGFP-PKWT and pDsRed-mutant).

Bioinformatics-based Study—A structural comparison of *E. coli* pyruvate kinase (Protein Data Bank code 1PYK) and human PKM₂ (Protein Data Bank code 1T5A) was carried out using FATCAT tool (flexible structure alignment by chaining aligned fragment pairs allowing twists) based upon a flexible and rigid model (23). The flexible model introduces twist in the loop region of protein for better alignment and minimum root mean square deviation (r.m.s.d.), whereas the rigid model tries to better align the two proteins without introducing any change in structure, like twist. The results were further validated by combinatorial extension method (24, 25). Sequence alignment of human and *E. coli* pyruvate kinase was done by using the ClustalW tool of EBI. The mutant structures were generated using the residue replace option in INSIGHT II. Hydrogens were added to the protein, and the same was subjected for energy minimization using CVFF force field in the discover module of INSIGHT II. Glide score was calculated to study the affinity for P-enolpyruvate. The stability of hetero-oligomers of mutant and wild type PKM₂ was calculated using FOLDX (26, 27) in terms of total free energy (\(G\); Gibbs free energy) assuming the system in water. The G value provides the conformational stability (free energy in kcal/mol) and predicts the overall stability of the protein. \(G\) represents the sum of the energy of all types of possible interactions within a protein molecule (backbone and side chain H bonds, van der Waal, electrostatics, solvation polar and hydrophobic, van der Waal and torsional clashes, entropy side chain, entropy main chain, helix dipole, water bridge, disulfide and the partial covalent bonds; lower the G value the higher the stability). The % probability of formation of all possible hetero-oligomers was calculated as a function of calculated free energy (\(G\)) using Equation 1,

\[
\% P_{x_1} = \left(G_{x_1}/\sum G_{x_1-x_n}\right) \times 100
\]  

(Eq. 1)

here, \(\% P_{x_1}\) represents the % probability of formation of \(x_1\) molecule, \(G_{x_1}\) represents the free energy of a molecule \(x_1\), and \(\sum G_{x_1-x_n}\) the total sum of free energy of all possible combinations (\(n\)) of the molecules. The chain-structure data of different combinations of wild type and mutant was generated using PdbSum.

RESULTS

Wild Type and Mutant PKM₂ Monomers Interact and Co-localize in Mammalian Cells—The absence of growth of the AH109 yeast strain expressing wild type or mutant PKM₂ fused with the GAL4 binding domain, in media lacking Trp and His, indicated that none of the wild type and mutant PKM₂ act as a transactivator. However, the cells co-expressing wild type and mutant PKM₂ fused with GAL4 activation and binding domains, respectively, grew well on triple dropout synthetically defined media (lacking His, Trp, and Leu) in the absence (Fig. 1A) and in the presence (Fig. 1B) of 20 mM 3-amino-1,2,4-triazole, indicating a positive interaction between wild type and mutant monomers of PKM₂. In the standard pulldown assay (as described under “Experimental Procedures”), the purified GST fused PK-WT was unable to pull out the His-tagged mutant proteins (data not shown); however, a positive interaction was observed only in case of the modified GST pulldown assay (as described under “Experimental Procedures”), where the wild type and mutant proteins were co-expressed in situ, essentially depicting the nature of protein interaction at the monomer level (Fig. 1C). An ~60-kDa His-tagged mutant protein was pulled down with the 86-kDa GST-fused PK-WT (Fig. 1C) that was substantiated by Western blot, using human PKM₂-specific monoclonal antibodies (supplemental Fig. S1). Confocal microscopy showed the co-localization of both the mutant proteins along with PK-WT in the cytoplasm of the mammalian cells (yellow dots), in addition to some formations of homotet-

FIGURE 1. A yeast two-hybrid assay showing the following: (1) yeast strain AH109 growing on triple dropout media lacking Leu, Trp, and His, co-transformed with pGBD-PK and pGAD-H391Y; (2) pGAD-PK and pGAD-H391Y; (3) pGAD-PK and pGBD-PK as a negative control; (4) control vector pGAD co-transformed with pGBD-PK as negative control, with no growth. B, repeated with media containing 20 mM 3-amino-1,2,4-triazole. C, modified GST pull-down assay. The elution of glutathione-agarose bead-bound fraction was loaded on 12% SDS-PAGE. GST-fused PKM₂ (86 kDa) pulled down “His-tagged” PK-WT, K422R, and H391Y (~60 kDa) mutants in lanes 2–4, respectively. Lane 5, negative control, showing the incapability of GST protein alone (26 kDa) to pull down the His-PKW. Lane 1, standard protein ladder (kDa). D, co-localization of wild type and two mutant PKM₂ proteins (upper and lower panels) in confocal microscopy. The cells were co-transfected with the independent clones expressing GFP-fused PK-WT and RFP-fused mutant protein (panels a and e), Hoechst-stained nucleus (panels b and f), GFP-tagged PK-WT (panels c and g), and RFP-tagged H391Y and K422R mutants, respectively (panels d and h), merged view.
Functional Implications of Human PKM$_2$ Mutations

Energy minimization study depicted that the wild type and mutant monomers have far more chances to interact with each other and generate stable hetero-oligomers (Fig. 2B). The percent probability of formation was calculated as a function of total free energy ($G$; interface stability of the monomers) and showed $\sim$5 and $\sim$10% probability for wild type homotetramer formation in case of co-expression of H391Y or K422R mutant with PK-WT, respectively. The mutant homotetramers depicted an average probability of almost 28%, whereas $\sim$65% probability of formation was shown by hetero-oligomers for both the mutants when co-expressed with wild type protein (Fig. 2C). The heterodimers of H391Y were also found to be 2.5 times more stable than homodimers of PK-WT (Fig. 2D). Glide score showed that heterodimers of both the mutants possessed lesser affinity for P-enolpyruvate in comparison with homodimers of PK-WT (supplemental Fig. S4). The stability of the mutant monomer was also found to be relatively less, increasing the chances to interact with wild type monomers to generate hetero-oligomers (Fig. 2E). These observations suggested that a mutation at the ISCD region of monomers is driving the cross-monomer interactions. The heterodimers of K422R and H391Y mutants with PK-WT were observed to possess 5 and 10 unique hydrogen bonds, respectively, at their ISCD region, explaining the relative stability of heterodimers in comparison with each other (supplemental Fig. S5).

Co-expression of Wild Type and Mutant PKM$_2$ Affects the Oligomeric State of PKM$_2$ and Generates Hetero-oligomers—E. coli cells expressing wild type and mutant PKM$_2$ simultaneously showed a substantial shift in the tetramer peak. The rightward shift in the peak indicated an elution of the low molecular weight dimeric form of PKM$_2$ from fractions 23–28. H391Y mutant showed a higher potential for dimer formation, in comparison with K422R mutant protein (Fig. 3A). The possibility of heterotetramer formation was analyzed by glutaraldehyde cross-linking assay, which confirmed the formation of heterodimers (146 kDa) of GST-fused wild type (86 kDa) and His-tagged mutant (~60 kDa) monomers; and also showed the cross-linked high molecular weight heterotetramers in the range of 240–344 kDa (Fig. 3B). Because the high molecular weight bands were not resolved efficiently enough despite many optimizations, we analyzed the presence of GST- and His-tagged heterotetramers with glycerol gradient. The difference in molecular weight reflected in the differentially positioned peaks (peaks 2–4) showed the potential of wild type-mutant “heterotetramer” formation with differential activity, depicted by activity peak intensity. A similar peak profile obtained in Fig. 3C represented the GST- and His-tagged wild type-wild type protein interaction. Peak marked “0” represented the heterodimer of wild type and mutant proteins. Peak 1 showed the outcome of the wild type and mutant co-expression, representing the His-tagged mutant monomer, lighter in weight (60 x 4 = 240 kDa), and peak 5 represented the homotetramer of GST-fused PKWT (86 x 4 = 344 kDa). However, peaks 2–4 represented the “intermediate/hybrid species,” representing the heterotetramers (Fig. 3, D and E). Peaks 1–5 in the case of GST- and His-tagged PKWT co-expression showed an equal probability of cross-monomer interaction.

Human and E. coli Pyruvate Kinase Share Significant Structural Similarity, and Wild Type and Mutant Human PKM$_2$ Hetero-oligomerization Is Energetically Favorable—Significant homology was observed in the nucleotide and protein sequences of human and E. coli PKM$_2$ (supplemental Figs. S2 and S3). A FATCAT analysis for higher ordered structural alignment showed 463 equivalent positions with an r.m.s.d. of 2.9 Å without introducing a twist. A twist is essentially a bend that the FATCAT tool introduces in one protein structure to align it better on another (flexible model). However, human and E. coli PK get aligned properly by the tool used without introducing any structural modification, like twist (rigid model), which depicts a greater homology. These results were substantiated using the combinatorial extension method which showed an r.m.s.d. of 2.5 Å with 454 aligned residues (r.m.s.d. value <3.5 Å represents a significant structure homology). However, only a few loop regions contributed maximally to the r.m.s.d. value observed (Fig. 2A). The secondary structure of two proteins was found to be almost similar (supplemental Table S2). A higher domain homology was also detected when the monomer of the two proteins was superimposed over each other (Fig. 2A).

FIGURE 2. A, superimposed monomers of human PKM$_2$ (red) and E. coli (green) pyruvate kinase showing significant homology. The arrow indicates the loop region, contributing maximally to the r.m.s.d. value. $G$ calculated Gibbs free energy ($G$) for all possible homo- and hetero-oligomers of wild type and mutant PKM$_2$. C, % probability of formation of each hetero- and homotetramers depending upon the Gibbs free energy value. D, Gibbs free energy calculation for heterodimers of PK-WT and two mutant proteins, H391Y and K422R. E, Gibbs free energy of A chain monomer in all possible combinations of hetero-oligomers for both the mutants.
A similar result was obtained in studies where HeLa cells (and other mammalian cells) co-expressing wild type and mutant types showed the presence of hetero-oligomers in the form of split peaks, although the dimer/tetramer ratio was relatively high in comparison with what was observed when co-expression was carried out in *E. coli* (supplemental Fig. S7–S9).

**Co-expression of Wild Type and Mutant PKM₂ Shows Altered Activity and Affinity**—The activity of PKM₂ reduced to 54% and 78% in *E. coli* cells co-expressing PK-WT with H391Y and K422R mutants, respectively (using pET28a(+) and pRSET-B expression vectors, respectively). The activity of PKM₂ reduced drastically to 34 ± 6% and 48 ± 4% in cells co-expressing H391Y and K422R mutants with PK-WT, respectively (Fig. 4A). Similar results were obtained in the case of mammalian cells overexpressing the wild type and mutant proteins (supplemental Fig S10). Affinity of PKM₂ from the cells co-expressing K422R mutant protein with PK-WT for substrate, P-enolpyruvate, was found to be reduced, with the $K_m$ value increasing to 0.698 ± 0.06 mM from 0.438 ± 0.04 mM and significantly increased for H391Y mutant when co-expressed with PK-WT, with a $K_m$ value of 0.146 ± 0.02 mM in comparison with PK-WT (0.438 ± 0.04 mM) (Fig. 4B).

**Co-expression of Wild Type and Mutant PKM₂ Accelerates the Bacterial and Mammalian Cell Growth Rate**—It was observed that the *E. coli* cells expressing mutant PKM₂ alone showed higher growth rate than wild type protein-expressing cells. The average doubling time was reduced to 13 and 14 min in the case of K422R- and H391Y-harboring cells, respectively. The average doubling time of *E. coli* was observed to be 20 min under standard conditions (28), as observed for untransfected and PK-WT-transfected *E. coli* cells in our case. However, the *E. coli* growth accelerated to a greater extent when both wild type and mutant proteins were co-expressed together, and the average doubling time reduced to 10 min for both the mutant proteins. The reduction in PKM₂ activity correlating with increased cell growth suggested that highly conserved protein has a similar effect across the species, because a mammalian protein affected the growth rate of the bacterial cells in response to the alerted PKM₂ activity (Fig. 5A).

After confirming the expression of transfected clones in HeLa cells by enzyme activity assay (supplemental Fig S10), it was observed that cells co-expressing the mutant proteins with PK-WT divided at a faster rate. The cells co-expressing K422R and H391Y mutant with PK-WT showed an ~10 and ~12 times higher number of cells, respectively, after 48 h of transfection (Fig. 5B). These observations were confirmed further by...
FACS study in which HT1080-N1 cells co-expressing K422R and H391Y mutants along with PK-WT showed an accelerated growth profile and 2–3-fold increased polyploidy in comparison with mock-transfected cells (Fig. 5, C–I, and Table 1).

DISCUSSION

The observation of two novel mutations in the ISCD region of PKM2 accounting for its reduced activity in BS cells (18, 19) followed the discovery of mutant BLM gene as an etiological factor for the syndrome (29). Incidentally, the defective BLM protein does not explain a number of clinical symptoms observed in the syndrome (30). Interestingly, 7% (9/134) of BS patients lack BLM mutations,3 and many available BS cell lines show down-regulated PKM2 activity.4 It was therefore pertinent to understand the mechanism of the down-regulation of PKM2 activity and its functional implication to infer the role the isozyme could play in a cell. We have proposed earlier that structural modulation in PKM2 mutant proteins may facilitate tumor survival, incidentally one of the key features of BS (20). However, it is not clear if a genetically inherent tendency for stabilization of the PKM2 dimer and other less active oligomeric forms of PKM2, as observed in this study, provides a unique way for activity regulation with a possible clue for genetic predisposition to cancer development in BS patients.

PKM2 Mutants Follow a Unique Pathway to Down-regulate Their Activities with Differential Affinity—The mechanism of alteration of PKM2 activity due to mutations was understood by yeast two-hybrid assay and confirmed by GST pulldown. The negative result in the “standard GST pulldown assay,” where the homotetrameric wild type as well as mutant proteins were mixed, provided the clue that the interaction was not simply a tetramer-tetramer association. However, co-expression of two proteins, mimicking in vivo heterozygous condition, provided a chance of protein-protein interaction at monomer level. Hence, a positive interaction observed only in a modified pulldown assay essentially depicted the hetero-oligomerization tendency of wild type and mutant proteins through cross-monomer mixing, a possible reflection of what happens in BS cells. In silico study provided an elementary clue about the existence of hetero-oligomers where the higher stability in terms of Gibbs free energy of each possible species, including mutant homotetramers, suggested an increased probability of their formation in cellular milieu. The proposed in silico hypothesis was validated by glycerol gradient experiments where a single peak of wild type as well as mutant PKM2 tetramer, when expressed independently (20) or in a natural condition in a cancer cell line (supplemental Fig. S6) (31), was found to split into five distinct peaks in accordance to their molecular weight (Fig. 3, C–E). The lower activity of GST-fused wild type homotetramers represented by peak 5 in the case of wild type-mutant co-expression (Fig. 3, D and E), depicted the reduced tendency for wild type homotetramerization. This corroborated with what was predicted in silico, suggesting that the stability of hetero-oligomers was high enough and left only a lower probability for homotetramer formation (Fig. 2C), which would have been 50% if the mutations were heterozygous and not dominant negative. It is likely that the higher stability of heterodimers is due to predicted extra unique H-bonds within the ISCD of wild type and mutant monomers, which also explains the higher tendency of H391Y mutant dimerization than the K422R mutant (supplemental Fig. S5). Incidentally, the higher dimer/tetramer ratio observed in the case of co-expression of wild type and mutant PKM2 in mammalian cells (supplemental Figs. S8 and S9), in comparison with the E. coli system, we suggest is a consequence of altered metabolic programming of the mammalian cells, essential for proliferation (32). All proliferating mammalian cells have a tendency to dimerize a significant portion of tetrameric PKM2 (supplemental Fig. S6) as observed earlier (14, 31), contributing positively to the mutation-driven tendency of dimerization in protein when expressed in the presence of PK-WT and an endogenous background expression of wild type PKM2. Nevertheless, the well resolved peaks

3 James German, personal communication.
4 A. Mammalapalli, N. Z. Baquer, and R. N. K. Bamezai, unpublished data.
representing tetrameric molecules depict the presence of heterooligomers (supplemental Figs. S7–S9) as observed in E. coli cells (Fig. 3, C–E), the latter representing a relatively advantageous unbiased model to study the natural tendency of heterooligomerization of wild type and mutant PKM2.

Allosteric proteins exist in a complex ensemble of conformers, where ligand binding to them does not induce conformational changes, rather it stabilizes the pre-existing active conformers to make them functional (33, 34). However, the altered affinity on binding to P-enolpyruvate, as observed (Fig. 4B),
leads to a differential stability and differential shift in the ensemble, which potentially altered the functional impact of PKM2 in the cell. Our previous study (20) showed that despite having mutations within the ISCD region, both the mutants showed differential impact over P-enolpyruvate affinity in comparison with PKWT. The increased affinity for P-enolpyruvate in the experiments of co-expression of H391Y with PKWT was due to its intrinsic environment of amino acid residue interaction as described previously (20). However, the co-expression of this mutant lost significant affinity when compared with its independent expression ($K_m = 0.078 \pm 0.009 \text{ mM}$) (20). Instead, the K422R mutant showed an increased affinity ($K_m = 0.698 \pm 0.06 \text{ mM}$) in comparison with its independent expression (1.5 mM $\pm 0.1$) but was significantly lower than the PKWT (0.146 $\pm 0.02 \text{ mM}$). The co-expressions of wild type and mutant proteins have a significant differential impact over the activity of proteins in comparison with their individual expression as observed earlier (20) and as a consequence of cross-monomer mixing. Fully active PKWT lost $\sim$60% of activity when expressed with H391Y mutant, which in turn is 80% active when expressed alone (Fig. 4A). The differential loss of activity is essentially a result of the sequestering of wild type monomers by mutant proteins (dominant negative effect), proved by modified GST pulldown and glycerol gradient assay. The reduced activity of hetero-oligomers observed as a result of heterogeneity of amino acid residues at the ISCD region rendered the signal transduction ineffective.

\textit{Co-expression of Wild Type and Mutant PKM2 Induces Accelerated Cell Proliferation and Polyploidy—Biological importance of the preferential hetero-oligomerization of PKM2} was reflected in the observation of enhanced cellular proliferation in bacterial and mammalian cells expressing the less active altered ensembles of PKM2. The reduction of PKM2 activity by subunit dissociation (dimerization) is known to block the glycolysis at the last step, allowing the accumulation of glycolytic intermediates. These intermediates are used as synthetic precursors at the time of cell division (14). The tumor incidentally needs a permanent supply of these intermediates; hence, permanent down-regulation of PKM2 activity by stable dimer formation provides a favorable condition for tumor development (14, 32, 35). Many oncogenic viral proteins are known to dimerize the PKM2 tetramer by physically interacting with it (36, 37), although recently, up-regulation of PKM2 expression and its activity down-regulation have been shown to be involved in \textit{in vivo} development of cancer (15, 38). The feature of stabilization of PKM2 dimers is observed in cancer cells (supplemental Fig. S6) and has been used as a diagnostic feature (39–41).

The expression of mutations in a heterozygous condition showed an inherent tendency of disturbing tetramer/dimer equilibrium by favoring the dimerization, therefore leading to accelerated cellular growth (Fig. 5, B–I, and Table 1), a prerequisite for cancer development. Although expression of PK-WT itself is a cell growth-promoting factor (Fig. 5, D and G), however, the mutant forms contribute to this phenomenon more than the wild type PKM2 (Fig. 5, E, F, H, and I). This in all probability is a result of relatively lowered activity and affinity of the hetero-oligomerized proteins, including the heterodimers. Polyploidy rate enhancement was an interesting observation that occurred in the cells co-expressing the mutant PKM2 along with the wild type proteins (Table 1), supporting the important role of the cross-monomer interactions of PKM2 and their reduced enzyme activity in sustaining neoplastic transformation and generating genomic instability as observed in BS (42). Incidentally, the increased growth rate of \textit{E. coli} as well as mammalian cells in response to the expression of a human protein (mutant PKM2) (Fig. 5A) indicates its evolutionary importance of highly conserved structure of PK from \textit{E. coli} to humans. Apparently, overexpressed human PKM2 shows a potential to replace the \textit{E. coli} pyruvate kinase in the bacterial glycolytic complex, suggesting that \textit{E. coli} could be used as a good model to study the effect of some conserved proteins and the role of their mutants.

The down-regulation of PKM2 activity had a direct co-relation with an increased cellular growth. The observation of H391Y mutant hetero-oligomer expression induces relatively more cellular growth and polyploidy than its individual expression, and K422R hetero-oligomer expression with lowered activity provides further support to our conclusion. Our previous observation of PKM2 deficiency in more than one BS cell line without any PKM2 mutation\(^6\) suggests that this down-regulation of the activity may happen irrespective of mutations in BS condition where stabilization of the PKM2 dimer or less active tetramers, through as yet unknown mechanisms, provides an inherent susceptibility to tumor development in early life in the syndrome.

\textit{Modulated PKM2 Can Justify Other Features of Bloom Syndrome, a Hypothesis—Human PKM2 has recently emerged as a multifunctional protein, and many of its nonglycolytic functions are little understood. A direct interaction of PKM2 with cytoplasmic promyelocytic leukemia tumor suppressor protein and the co-localization of BLM with promyelocytic leukemia in maintaining genome stability (31, 43) indicate an indirect role of PKM2 in genome stability. The observation of an increased rate of polyploidy in this study opens up an area of investigation in this direction. Reports have also shown the involvement of

### Table 1

**Effect of wild and mutant PKM2 co-expression on the cell cycle progression**

A percentage of the cell population was analyzed in all phases of the cell cycle after 48 h of co-transfection of GFP-wild and RFP-wild (PKWT-PKWT), GFP-wild and RFP-H391Y mutant (PKWT-H391Y). Both wild and mutant proteins were also co-expressed using single bidirectional vector (pbi-Tet).

| Phase     | Mock | PKWT-PKWT | PKWT-K422R | PKWT-H391Y | pbi-PKWT-PKWT | pbi-PKWT-K422R | pbi-PKWT-H391Y |
|-----------|------|-----------|------------|------------|---------------|----------------|----------------|
| G1        | 55 ± 5 | 45 ± 6 | 39 ± 6 | 34 ± 5 | 43 ± 4 | 40 ± 6 | 40 ± 4 |
| S         | 5 ± 1 | 7 ± 2 | 6 ± 3 | 9 ± 1 | 6 ± 1 | 6 ± 1 | 6 ± 1 |
| G2        | 20 ± 2 | 19 ± 3 | 16 ± 3 | 12 ± 5 | 16 ± 3 | 15 ± 3 | 13 ± 2 |
| Polyploidy| 13 ± 3 | 23 ± 4 | 30 ± 3 | 37 ± 5 | 26 ± 7 | 30 ± 4 | 36 ± 3 |
Functional Implications of Human PKM₂ Mutations

pyruvate kinase polymorphism in association with type II diabetes (44). We propose that an activity-deficient PKM₂ would not metabolize glucose despite having enough insulin in the blood, thus providing a reason for the occurrence of a type II diabetes-like situation observed in some BS patients. A defective PKM₂ has the potential to create an imbalance in the equilibrium in growth and apoptosis (45, 46) thus resulting in growth retardation in BS. Similarly, a defective sperm-specific pyruvate kinase would be unable to maintain normal glycolysis, crucial for sperm mobility (47, 48), to explain male-specific infertility in BS. Also, PKM₂ has been reported to interact with microtubules physically (49), with the possibility to affect the motility of microtubule-rich cells like sperm cells. The feature of facial erythema observed in BS patients again could be explained by an allergic reaction due to PKM₂-dependent “mast cell degranulation,” responsible for hypersensitivity (50).

Conclusion—A large number of multifunctional oligomeric proteins in evolution have emerged to handle multiple functions simultaneously; however, sometime their defective status results in susceptibility to a particular disease. An important glycolytic protein, the PKM₂ has turned out to be one such candidate, involved not only in glycolysis but also in a variety of cellular functions. We have shown that the down-regulation of PKM₂ activity is due to an inherent tendency to express as dimers or other less active forms and is directly correlated with an increased rate of cell cycle and polyploidy, the hallmark of cancer cells, the feature common to BS patients. This mimics the heterozygous status of this gene in Bloom syndrome conditions. This study not only signifies the importance of highly conserved amino acid residues at critical positions that can aid one to understand the long range communication in allosteric proteins (33), but also indicates the potential therapeutic role of PKM₂ by supplementing the normal PKM₂. After all, curing of BS cellular features through BLM transfection (51) and simple co-cultivation with normal cells (52, 53) in in vitro studies have indicated such a potential.

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