Phosphoglycerate mutase-derived polypeptide inhibits glycolytic flux and induces cell growth arrest in tumor cell lines

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Running title:

Growth arrest by PGM inhibition in MCF-7 cells
Summary

The putative tumor metastasis suppressor protein Nm23-H1 is a nucleoside diphosphate kinase which exhibits a novel protein kinase activity when bound to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In this study we show that the glycolytic enzyme phosphoglycerate mutase B (PGM) becomes phosphorylated in the presence of the Nm23-H1/GAPDH complex in vitro. Mutation of His10 in PGM abolishes the Nm23-H1/GAPDH complex-induced phosphorylation. Nm23-H1, GAPDH and PGM are known to co-localize as shown by free flow isoelectric focusing. In association with Nm23-H1 and GAPDH, PGM could be activated by dCTP which is a substrate of Nm23-H1, in addition to the well-known PGM activator 2,3-bisphosphoglycerate. A synthetic cell penetrating peptide (PGMtide) encompassing the phosphorylated histidine and several residues from PGM (LIRHGE) promoted growth arrest of several tumor cell lines, whereas proliferation of tested non-tumor cells was not influenced. Analysis of metabolic activity of one of the tumor cell lines, MCF-7, indicated that PGMtide inhibited glycolytic flux, consistent with in vivo inhibition of PGM. The specificity of the observed effect was further determined experimentally by testing the effect of PGMtide on cells growing in the presence of pyruvate, which helps to compensate PGM inhibition in the glycolytic pathway. Thus, growth of MCF-7 cells was not arrested by PGMtide in the presence of pyruvate. The data presented here provide evidence that inhibition of PGM activity can be achieved by exogeneous addition of a polypeptide, resulting in inhibition of glycolysis and cell growth arrest in cell culture.
Introduction

Phosphoglycerate mutase (PGM, E.C. 5.4.2.1) is an enzyme of the glycolytic pathway where it catalyses the conversion of 3-phosphoglycerate to 2-phosphoglycerate. While this step is not considered to be rate limiting in the majority of differentiated cells, there is increasing evidence from tumor cell lines that PGM may regulate the balance between glycolysis and another ATP-producing pathway, glutaminolysis (1-5). Besides in tumor cells a rate-limiting role of PGM within the glycolytic pathway has also been shown in leucocytes and heart muscle (6, 7). PGM is activated by the cofactor 2,3-bisphosphoglycerate (2,3-BPG) which phosphorylates the enzyme at histidine. In tumor cells PGM is regulated by migration out of the glycolytic enzyme complex into the so-called pre-complex. The PGM associated within the glycolytic enzyme complex and within the pre-complex can be separated by free flow isoelectric focusing (4, 5, 8, 9). The PGM enzyme associated within the glycolytic enzyme complex is fully activated and independent of 2,3-BPG whereas the PGM enzyme found in the pre-complex is not fully active and can be activated by 2,3-BPG (4, 5, 9). In the pre-complex PGM is in close proximity to nucleoside diphosphate kinase type A (E.C. 2.7.4.6; NDPK A) (5, 9). Migration of PGM out of the glycolytic enzyme complex into the pre-complex reduces the conversion of glucose to lactate and increases the flux rate through glutaminolysis as well as serine synthesis. In addition, ATP and GTP levels decrease whereas UTP and CTP levels stay high. A low (ATP + GTP) : (UTP + CTP) ratio is correlated with a high proliferation rate (10-13). NDPK directly couples ATP levels with other nucleoside triphosphates (5, 9, 12, 13). NDPK demonstrates a “ping-pong” mechanism of catalysis and thus, upon incubation with NTP-Mg, the enzyme becomes phosphorylated at a histidine residue, forming a relatively stable phosphohistidine intermediate. The phosphate can then be transferred to a NDP-Mg acceptor.
Research on NDPK was stimulated when the cDNA of NDPK A was cloned as a transcript with reduced expression levels in tumor metastatic cells, termed Nm23-H1 gene (14). Eight related genes have been thus far identified as members of the human Nm23 gene family (15). The only corresponding gene products that have been well characterized are Nm23-H1 and Nm23-H2, which encode NDPK A and B, respectively. In addition to their main function of phosphorylating nucleoside diphosphates both isoforms show different additional biochemical activities (16-18). Nm23/NDPK protein isoforms have been reported to interact with a variety of cellular proteins and were found to be associated with different cellular compartments (17, 18). In terms of alternative biochemical functions, Nm23-H2 was found to be a transcriptional activator for the c-myc proto-oncogene (19-21). Nm23 has also been demonstrated to exhibit a protein kinase activity in vitro (22-26). In previous studies we have shown that PGM and Nm23-H1 (NDPK A) are associated and that the Nm23-H1 histidine kinase function is activated by association with GAPDH (5, 9, 23).

In the present study, PGM was identified as a protein phosphorylated by the Nm23-H1/GAPDH complex in vitro. Based on the phosphorylation site sequence, we developed a cell permeable synthetic peptide that encompasses the PGM phosphorylation site and inhibits PGM activity as well as glycolysis in MCF-7 cells and other tumor cell lines.
Experimental procedures

Expression of recombinant proteins and purification

The (His)\textsubscript{6}-Nm23-H1/GAPDH complex was expressed from a bicistronic baculovirus vector in Sf9 insect cells and purified by affinity chromatography on Ni\textsuperscript{2+} agarose followed by pseudo-affinity chromatography on reactive yellow agarose (Sigma Chemicals) as described earlier (23). The complete coding region of human PGM B was PCR-amplified from human kidney parenchyma cDNA and cloned into the BamHI/HindIII restriction sites of the pQE30 expression vector (Qiagen), using the following primers: 5´-taggatccatggccgcctacaaactg-3´ (forward) and 5´-cgaagcttaatgaaaatgggagccactg-3´ (reverse). After sequencing to ensure that the inserted sequence is correct, the plasmid was used to transform E.coli JM109, the bacteria grown at 37°C to an OD\textsubscript{600} of 0.5 and the expression induced by addition of 0.5 mM IPTG for three hours. The bacteria were harvested by centrifugation, lysed and the recombinant PGM purified by Ni\textsuperscript{2+} affinity chromatography under exactly the same conditions as described earlier for other his-tagged proteins (23). In a second step, the PGM eluted from the Ni\textsuperscript{2+} agarose column was dialyzed overnight against 20 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4} buffer pH 7.1, 0.5 mM DTT at 4°C. The protein was then passed over a hydroxylapatite column (BioRad) previously equilibrated with the same buffer. Bound proteins were eluted using a linear gradient from 20 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4} buffer to 500 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4} buffer. The fractions containing PGM were pooled, dialyzed against storage buffer (20 mM Tris/HCl, pH 7.5, 40% glycerol, 40 mM NaCl, 1 mM DTT, 0.5 mM EDTA) and stored at −20°C.

To generate the PGM\textsuperscript{His\textsubscript{10}Gly} mutant protein, site directed mutagenesis was performed using the plasmid carrying the wild type sequence and the following mutagenesis primers: 5´-gtgctgtgatc ggggcggccagagegca-3´ (forward) and 5´-tcgctctgccgccccgatgacac-3´ (reverse).
Mutagenesis was done using the QuikChange™ kit from Stratagene according to the manufacturer’s instructions. Expression and purification of the PGM^{His10Gly} mutant protein were carried out exactly as with the wild type protein.

**Screening of a cDNA expression library by in situ-phosphorylation**

To identify *in vitro* substrates of the Nm23-H1/GAPDH complex, a λ-TripleEx fetal brain cDNA expression library (Clontech) was screened. The phages were plated in LB top agar containing E.coli host cells and grown until plaques became slightly visible. Phages (10,000 p.f.u./plate) were blotted for 4 h at 37°C onto nitrocellulose filter membranes (Duralose-UV™, Stratagene) previously soaked in 10 mM IPTG. In addition, the plates were left with the membranes at 4°C overnight. The nitrocellulose sheets were then removed, washed twice in 20 mM Tris/HCl, pH 7.0, 150 mM NaCl, and blocked in 20 mM Tris/HCl, pH 7.0, 150 mM NaCl, 1 mM GTP, 2% BSA. Filters were then incubated with recombinant GAPDH/Nm23-H1 complex (0.1 mg/ml) in the presence of 15 µM $\gamma^{32}$P-GTP (0.37 MBq/ml) in 20 mM Tris/HCl, pH 7.0, 40 mM NaCl, 1 mg/ml BSA for 40 min at 30°C in roller bottles. After washing three times with large volumes of ice-cold wash buffer (20 mM Tris/HCl, pH 7.0, 150 mM NaCl, 0.1 % NP-40) for 5 min each, membranes were incubated in the same buffer containing 1 mM GDP for 10 min at room temperature under shaking to attenuate autophosphorylation of library-encoded NDPK isoenzymes. Filter membranes were washed again in ice-cold wash buffer and exposed wet to film for 2-4 days using intensifying screens at -80°C. After the first autoradiography, nitrocellulose membranes that produced signals were subjected to acidic buffer treatment (0.2 M glycine/HCl, pH 1.5) and exposed to film again. The phage plaques corresponding to radioactive spots were excised from the plates, eluted and used for a second round of plating and *in situ*-phosphorylation screening. Only phages corresponding to autoradiographic spots that were abolished after the acidic washing step in the first round continued to produce signals in the secondary screenings. Single phage
clones were excised from the agar plates and the cDNA inserts amplified by PCR, using the LD-insert screening amplimer set (Clontech, Cat. No. 9107-1). PCR products were subcloned into pCR4.1 TOPO cloning vectors (Invitrogen) and the plasmids sequenced.

**Phosphorylation of PGM by the Nm23-H1/GAPDH complex**

If not stated otherwise, phosphorylation assays were performed using 1 µg recombinant PGM and 200 ng Nm23-H1/GAPDH complex in a final volume of 25 µl in the following buffer: 20 mM Tris/HCl, pH 7.2, 40 mM NaCl, 5 mM MgCl₂, 1 mM DTT. Reactions were started by the addition of $\gamma^{32}$P-GTP or $\gamma^{32}$P-ATP to a final concentration of 20 µM (1 µCi per reaction). After incubation at 30°C for 10 min, 2 mM cold ATP was added to the samples to decrease the content of $^{32}$P-histidine phosphorylated Nm23-H1, followed by SDS sample buffer pH 8.8 after a 5 min incubation. The samples were immediately applied to SDS polyacrylamide gel electrophoresis without prior heating. These precautions were taken to avoid artificial chemical transfer reactions. The SDS gels were prepared according to Laemmli except that Tris/HCl buffer pH 8.8 was used for both stacking and separating gels. After electrophoresis, the gels were dried without prior fixation at 80°C under vacuum and autoradiographed overnight without intensifying screens.

For the determination of phosphate incorporation into PGM, phosphorylation was stopped after incubation with $\gamma^{32}$P-GTP by snap freezing in liquid nitrogen. Before re-thawing, the solution was adjusted to 8M urea, and paramagnetic beads loaded with Ni²⁺ were added to the samples to bind his-tagged proteins (PGM and GAPDH). Beads were washed quickly four times with a cold buffer consisting of 50 mM NaH₂PO₄/Na₂HPO₄, pH 8.5, 6 M urea, and counted in a liquid scintillation counter for $^{32}$P content. Phosphate incorporation per mol PGM was calculated based on counts of known amounts of $^{32}$P-GTP measured in parallel. To calculate possible background phosphorylation of his-tagged GAPDH, samples which had
been prepared and incubated exactly as the PGM phosphorylation reactions except that PGM was omitted were also counted.

**Peptide synthesis**

The PGMtide peptide (MRQIKIWFPNRRMKWKKHHHHHPWLIRHGE) and the control peptide (MRQIKIWFPNRRMKWKKHHHHHPWRIEGHL) were synthesized by ThermoHybaid (formerly Interactiva), Germany. N-terminally fluoresceine-coupled derivatives of these peptides and of another peptide lacking the Antennapedia domain (HHHHHHPWLIRHGE) were also synthesized. The freeze-dried peptides were dissolved in phosphate buffered saline containing 5 M urea. In the experiments, the peptides were added to cells by rapid dilution into the fresh culture media.

**Isolation of the glycolytic enzyme complex by isoelectric focusing**

Cells were extracted with a homogenisation buffer containing 10 mM Tris, 1 mM NaF and 1 mM mercaptoethanol, pH 7.4. Isoelectric focusing was carried out with a linear gradient of glycerol (50% to 0% (v/v)) and ampholines (pI 3.5-10.5) as described previously (8).

**Measurements of enzyme activities**

Specific activities of PGM, NDPK and GAPDH were measured as described earlier (8, 23, 27). For the heat inactivation assay, PGM was heated to 80°C in 50 µl of a buffer containing 50 mM Tris/HCl pH 7.2, 10 mM DTT and 0.1% NP40, then chilled on ice.

**Cell culture**

MCF-7 and Tx3095 cell lines, rheumatoid synoviocytes and amniotic fluid cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Deisenhofen, Germany), supplemented with 5 mM glucose, 2 mM glutamine, 100 units penicillin/ml, 100 µg
streptomycin/ml and 10% foetal calf serum (FCS). The medium also contained 4 mM sodium pyruvate if not otherwise stated. GLC4 cells were grown in RPMI-1640 medium supplemented as described for MCF-7 and Tx3095 cells. Cells were grown in a humidified atmosphere under 95% air/5% CO₂. MDA-MB-453 cells were grown in Leibovitz L15 medium supplemented with 2 mM glutamine, 4 mM pyruvate, 100 units penicillin/ml, 100 µg streptomycin/ml and 10% fetal calf serum (FCS). Cells were grown at 37°C in air-tight closed culture flasks.

The breast carcinoma cell lines MCF-7 and MDA-MB-453 were obtained from the ATCC, the small cell lung carcinoma cell line GLC4 was a gift from the Department of Internal Medicine, University Hospital Groningen, The Netherlands. The glioblastoma cell line Tx3095 was cultivated over 68 passages from a resected glioblastoma in the Department of Human Genetics, Homburg. Human amniotic fluid cells had been cultivated and used before for diagnostic purposes at the Department of Human Genetics, Homburg. Rheumatoid synoviocytes were obtained from a patient who underwent therapeutic synovectomy at the Department of Orthopaedics, University Hospital, Homburg.

**Fluorescence microscopy using fluorescein-linked peptides**

Fluorescein-linked versions of the peptides were added to the fully supplemented medium of MCF-7 cells grown directly on glass slides. After an incubation for 10 minutes at 37°C, the cells were washed three times with medium, fixed for 5 min in PBS/4% paraformaldehyde at room temperature, washed three times in PBS, counterstained with DAPI solution, washed again two times with PBS, dried, and mounted with antifade solution (Vectorshield; Vector Laboratories, Burlingame, CA, U.S.A.) for examination using FITC and DAPI filters.
Glycolytic flux measurements

Cell culture supernatants were collected at different cell densities and immediately frozen in liquid nitrogen. Glucose, pyruvate, lactate, glutamine, glutamate, serine and alanine were measured in the thawed supernatants as described previously (27-29). For the determination of the metabolite conversion rates two different calculations were chosen: The calculation in nmoles/(h*10^5 cells) reflects the conversion rate of a number of cells. The calculation in nmoles/ (h * dish) was chosen for the correlation of different conversion rates.
Results

Identification of protein(s) phosphorylated by the Nm23-H1/GAPDH complex in vitro

To elucidate possible functions of the Nm23-H1/GAPDH protein kinase activity, an attempt was made to identify potential substrate proteins. For this purpose, a cDNA expression library in λ-phages was transferred to nitrocellulose filters and screened by incubation with the bacterially expressed Nm23-H1/GAPDH protein complex in the presence of $\gamma^{32}$P-GTP-Mg. After Autoradiography of the filters, few isolated radiolabelled spots were detected on the films (see example in Fig. 1A, marked by arrow) and the corresponding phage plaques picked from the primary plates. After the first autoradiography, phosphorylation signals were further analyzed by incubating the membranes in acidic buffer (glycine/ HCl pH 1.5) and re-exposing to film. Using this procedure, acid labile phage phosphorylations could be detected and further discriminated from background spots. No true acid stable phage phosphorylation signals, indicative of serine, threonine or tyrosine phosphate, were found. Phage plaques having produced acid labile phosphorylations were picked and used for a second round of plating and screening. On the autoradiograph, the number of phosphorylated spots corresponding to phage plaques had increased (Fig. 1B), suggesting enrichment of phages displaying a phosphorylation substrate protein. The cDNA inserts of selected phage clones from the second-round plates were amplified by PCR and sequenced (marked by arrows in Fig. 1B). A BLAST search with the sequences revealed that among six non-redundant clones obtained from several plates, four contained the full-length coding sequence of human PGM B, while the two remaining clones contained 5'-cDNA sequences from PGM B of different length. The consensus coding sequences were identical to the gene bank entry for PGM B cDNA (accession number BC010038.1).
Characterization of the phosphorylation and identification of the phosphoamino acid phosphorylated by Nm23-H1

The complete coding sequence of PGM B was amplified by PCR. The PCR product was cloned into an expression vector, and the corresponding protein was expressed in E.coli and purified to homogeneity. The purified protein was phosphorylated in the presence of Nm23-H1/GAPDH complex and $\gamma^{32}\text{P-ATP}$ or $\gamma^{32}\text{P-GTP}$ but not in the presence of $\gamma^{32}\text{P-ATP}$ or $\gamma^{32}\text{P-GTP}$ alone (Fig. 2A). PGM was phosphorylated as a native protein as well as after denaturation by heat treatment. Since the heat treatment for 2 min at 80 °C was sufficient to completely inactivate PGM, as verified by activity measurements (Table I), this experiment excludes the possibility that PGM activity could be required for the observed phosphorylation.

Stoichiometric analysis showed that less than 0.1 mol phosphate was incorporated per mol PGM after 1h incubation time. However, maximum phosphorylation was achieved already after 1 min incubation (Fig. 3).

To be active, PGM normally requires small amounts of the co-factor 2,3-BPG, which serves as a phosphate donor for the initial activating autophosphorylation of the enzyme at histidine-10 at the active site. To determine whether the same residue might be targeted by Nm23-H1, histidine-10 was replaced by glycine using site-directed mutagenesis and the mutant protein bacterially expressed and purified. By this means it was possible to show that the his10-gly mutant was not phosphorylated by Nm23-H1/GAPDH (Fig. 2B), suggesting that it may be the phosphorylation site targeted by Nm23-H1/GAPDH in vitro.

Dependence of Nm23-H1-induced PGM activation on a native protein complex environment

Possible effects of the in vitro-phosphorylation of PGM were then investigated. Hypothetically, Nm23-H1-dependent phosphorylation might activate dephosphorylated PGM in a similar manner to 2,3-BPG. However, we were unable to verify any effect of Nm23-H1...
or Nm23-H1/GAPDH on purified PGM in vitro, possibly due to the low stoichiometry of phosphorylation. We hypothesized that the correct ratio and/or concentration of Nm23-H1/GAPDH and PGM as well as a defined orientation of the proteins in a complex environment might be essential for an interaction of Nm23-H1/GAPDH with PGM. To investigate Nm23-H1 involvement in PGM activation in the native complex, cytoplasmic proteins were extracted from proliferating MCF-7 cells and fractionated by isoelectric focusing, which allows separation of the so called glycolytic enzyme complex which contains most of the glycolytic enzymes and the so called pre-complex which contains PGM, NDPK, GAPDH as well as the cytosolic isoenzymes of the transaminases and glutamate dehydrogenase (Fig. 4A).

Analysis of the isoelectric focusing fractions of the MCF-7 cells revealed that the PGM enzyme associated within the pre-complex (Fig. 4B, fractions 33-37) could be activated by 2,3-BPG whereas the PGM enzyme associated within the glycolytic enzyme complex was only weakly activated (Fig. 4B, fractions 38-43).

As described earlier (30), addition of 0.5 mM orthovanadate into the enzyme assay leads to a complete inactivation of PGM in the absence of stoichiometric amounts of 2,3-BPG. Under these conditions, PGM activity is only measurable when the enzyme is permanently re-phosphorylated and thus re-activated by 2,3-BPG. In our study, in the presence of vanadate, a strong activation of PGM by 2,3-BPG was detected in the fractions of the pre-complex (Fig. 4C, fractions 33-37) whereas the PGM enzyme associated within the glycolytic enzyme complex was not activated (Fig. 4C, fractions 38-43). Most importantly, in the fractions of the pre-complex PGM was also activated by dCTP, whereas dCTP did not activate the PGM enzyme within the fractions of the glycolytic enzyme complex (Fig. 4C, triangles). These data show that in the pre-complex which contains Nm23-H1 and GAPDH, dCTP can substitute for 2,3-BPG as a PGM activating agent.
Effect of the PGMtide peptide on the Nm23-H1-induced phosphorylation and activation of PGM

If activation of PGM in tumor cells occurs by a direct phosphotransfer from Nm23-H1 protein, a synthetic peptide containing the phosphorylation site sequence for the in vitro-phosphorylation should be competitive in this reaction by acting as an alternative substrate – both in vitro and in vivo.

Therefore, the sequence LIRHGE at the C-terminus of PGM surrounding the phosphorylated histidine-10 residue was fused to an Antennapedia homeobox peptide sequence from Drosophila to enable cell penetration of the peptide in cellular assays. In vitro, the peptide could inhibit the phosphorylation of full-length PGM by Nm23-H1/GAPDH effectively at a concentration of 2 µM. Furthermore, we could verify that the peptide becomes phosphorylated (Fig. 5). Therefore, the inhibition could be due to competition for the substrate. No such phosphorylation inhibition was observed using a synthetic control peptide, which was identical to PGMtide except that the amino acids derived from the PGM sequence were in a random order (RIEGHL instead of LIRHGE). The specificity was further highlighted by the fact that the control peptide was not phosphorylated by Nm23-H1/GAPDH. Further characterisation indicated that PGMtide (in concentrations up to 20 µM) did not inhibit the isolated PGM activity in the presence of 2,3-BPG nor did it influence NDPK activity of the Nm23-H1 protein in the purified Nm23-H1/GAPDH complex (Fig 6).

The ability of PGMtide and the control peptide to enter the cytoplasm of MCF-7 cells was ascertained by using fluoresceine-labelled variants of the synthetic peptides (Fig. 7). Ten minutes after addition of the peptides to the culture medium, a strong cytoplasmic fluorescence signal could be detected for both the fluorescein-PGMtide (Fig. 7A) and the fluorescein-control peptide with the PGM-derived amino acids in changed order (RIEGHL instead of LIRHGE) (Fig. 7B). In contrast, only DAPI-stained nuclei but no cytoplasmic
fluorescence could be observed in the MCF-7 cells when a fluorescein-labelled PGMtide version lacking the Antennapedia homeodomain sequence was used (Fig. 7C).

Effect of PGM peptides on cell proliferation and glycolytic rates of MCF-7 cells cultivated in the absence of pyruvate.

For metabolic measurements, the cell line MCF-7 was chosen because it shows a high glycolytic rate. In addition, the metabolic pathways of phosphometabolites have been thoroughly characterized (28, 29). The cells were treated with non-cytotoxic concentrations (7 μM) of both PGMtide and control peptide. The cells and the culture supernatants were harvested at different time points. Harvested cells were counted as a measure of cell growth. The supernatants were used to determine concentrations of key metabolites of the glycolytic and glutaminolytic pathway. The growth diagram of MCF-7 cells cultivated in the absence of pyruvate (Fig. 8A) shows that a single application of PGMtide caused cell growth arrest for about two days of cultivation. After two days, the cells resumed growth, indicating that the effect of the peptide was transient under these conditions, probably due to degradation of the PGMtide. The peptide’s effect was most obvious between 24 h and 48 h of incubation with PGMtide, reducing MCF-7 cell growth by more than 10-fold in comparison to control-treated cells (Fig. 8C).

In control peptide-treated MCF-7 cells serine concentrations in the medium remained almost unchanged with increasing rates of glucose consumption (Fig. 9A, slope = 0.002). In PGMtide-treated MCF-7 cells serine production increased with increasing glucose consumption (Fig. 9A, slope = 0.19). This result indicates that 3-phosphoglycerate was used for serine synthesis, due to an inhibition of PGM by the PGMtide (Fig. 10).

The amino group which is used for serine synthesis derives from glutamine. Accordingly, glutamine consumption by MCF-7 cells is significantly increased in the presence of the PGMtide (Fig. 9D). In the absence of extracellular pyruvate, Glutamine
consumption correlates with lactate production, indicating that glutamine is finally converted to lactate (Figs. 9D, 9E). Since the glutamine amino group is either transferred to 3-phosphohydroxypyruvate (derived from 3-phosphoglycerate) to form serine or to pyruvate to form alanine, the rates of synthesis of both amino acids are coupled with glutaminolysis (Fig. 10). In the presence of the control peptide, serine production positively correlated with alanine production (Fig. 9B; slope = +2.14). This indicates that in the presence of the control peptide sufficient levels of 3-phosphoglycerate and pyruvate are available for serine and alanine synthesis, respectively. The correlation between serine production and alanine production dramatically changed upon treatment with PGMtide (Fig. 9B; slope = -8.56). The negative correlation indicates that alanine production is inhibited at high serine production rates. 3-phosphoglycerate is mainly converted to serine and not to pyruvate (Fig. 10).

Effect of extracellular pyruvate on the PGMtide-induced inhibition of cell proliferation

Since PGMtide reduced the availability of pyruvate in MCF-7 cells, we investigated whether the PGMtide-induced inhibition of cell proliferation can be overcome by extracellular pyruvate. When the PGMtide-treated MCF-7 cells were cultivated in the presence of extracellular pyruvate, lactate production increased with increasing glucose consumption whereas no correlation was found between these two parameters when the cells were cultivated in the absence of extracellular pyruvate (Fig. 9C). Accordingly, the cell proliferation rate of MCF-7 cells increased when pyruvate was added to the cultivation medium (Figs. 8A, B).

Furthermore, in PGMtide-treated cells extracellular pyruvate led to a dramatic reduction of glutamine consumption as well as of the flow of glutamine to lactate in the PGMtide-treated cells (Figs. 9D, 9E).

Anti-proliferative effects of PGMtide on other cell types
The effect of PGMtide was not limited to MCF-7 cells. As already shown above, growth inhibition was also promoted in another breast carcinoma cell line, MDA-MB-453 (Fig. 8C). In contrast to MCF-7 cells however, in MDA-MB-453 cells PGMtide-induced growth inhibition was not abolished by exogenous pyruvate.

Furthermore, PGMtide inhibited the proliferation of several other tumor cell lines, such as the glioblastoma cell line Tx3095 and the small cell lung cancer cell line GLC4, when tested in a concentration range between 5 and 15 µM (Fig. 11). The control peptide was without visible effect in each case. Among the tested cell lines, GLC4 was most responsive and displayed cell death at PGMtide concentrations above 7 µM (Fig. 11A). However, after withdrawal of PGMtide from the medium, surviving cells continued to grow normally.

For comparison, PGMtide was also tested with primary cells of non-tumor origin, including amniotic fluid cells and rheumatoid synoviocytes; the peptide did not influence neither proliferation nor viability of those non-tumor cells (Figs. 11C, D).
Discussion

It has long been recognised that the glycolytic pathway is important for tumor growth especially under hypoxic conditions (13, 31). The glucose carbons can be degraded to lactate with a concomitant production of energy or can be channelled into synthetic processes, such as nucleic acid, lipid and amino acid synthesis (32, 33). Since synthetic processes are energy consuming the use of glucose carbons for either energy production or for synthetic processes must be balanced exactly, especially in solid tumors which are under the constraints of extreme fluctuations in glucose and oxygen supply. This may be the reason why tumor cells exploit glutamine as an alternate energy source (1-5). Glutamine is available in high concentrations in all tissues. The interaction between glucose and glutamine consumption is in part controlled by the migration of PGM outside the glycolytic enzyme complex into the so-called pre-complex (4, 5, 9), which correlates with an increased conversion of 3-phosphoglycerate to serine. Serine is an essential precursor for nucleic acid and phospholipid synthesis in that it supplies one-carbon fragments needed in this process (Fig. 10). In addition the migration of PGM out of the glycolytic enzyme complex leads to an increased glutamine consumption as well as a reduced pyruvate and alanine production (5, 9). Purified PGM enzymes of all vertebrates require the co-factor 2,3-BPG for activity (34). Differentiated tissues such as liver, brain and erythrocytes are characterized by high levels of 2,3-BPG whereas in proliferating cells, 2,3-BPG is only present in spurious amounts and is undetectable in tumor cells. Tumor formation is correlated with a reduction of the 2,3-BPG synthesizing enzyme 2,3-bisphosphoglycerate mutase (E.C. 5.4.2.4) (37-41). Since tumor cells are characterized by high glycolytic rates although 2,3-BPG is not detectable, another 2,3-BPG-independent PGM activating mechanism can be postulated.

In the present study we have identified PGM as a new substrate for Nm23-H1/GAPDH histidine kinase activity in vitro. All three proteins, GAPDH, Nm23-H1, and
PGM co-localise together within the glycolytic pre-complex (Fig. 4A) (4, 5, 9). The novel function described in this report appears to be exerted by the Nm23-H1 protein, but not by the close homologue Nm23-H2, since only the acidic isoform of NDPK, which is NDPK type A (Nm23-H1), was found in the pre-complex. The Nm23-H1 dimer which is able to phosphorylate PGM is stabilized by binding to GAPDH (23).

Histidine kinase activity of Nm23/NDP kinase on several substrate proteins including ATP citrate lyase, succinic thiokinase and G protein β-subunits in vitro was reported independently by several groups (24-26, 35, 36, reviewed in 16). In accordance with an involvement of Nm23-H1/NDPK, PGM in the glycolytic pre-complex could be activated in vitro by dCTP, a nucleotide which is very unlikely to be used as a phosphate donor by other kinases (Fig. 4C). The phosphorylation of PGM by NDPK/GAPDH was independent of PGM activity since it proceeded in the presence of a heat-denatured form of PGM (Fig. 2A). This suggests that the recognition motifs for PGM phosphorylation by Nm23-H1/GAPDH may be in a short contiguous linear stretch of the PGM primary sequence. A synthetic cell permeable polypeptide (PGMtide) encompassing a sequence of PGM containing the histidine phosphorylation site was therefore generated, which inhibited phosphotransfer from Nm23-H1 to purified PGM (Fig. 5) and also specifically suppressed growth of tumor cells in culture (Fig. 11).

The treatment of MCF-7 cells with PGMtide mimicked the metabolic effect of the PGM migration out of the glycolytic enzyme complex (Figs. 9A-E). The fact that tumor cells were more sensitive to PGMtide-induced inhibition of cell proliferation than normal proliferating cells can be explained by a per se reduced flow of 3-phosphoglycerate to 2-phosphoglycerate in tumor cells due to the migration of PGM out of the glycolytic enzyme complex (4, 5, 9).
The result that in MCF-7 cells, extracellular pyruvate compensates the inhibitory effect of the PGMtide demonstrates that the PGMtide-induced inhibition of cell proliferation is specifically caused by an inhibition of a glycolytic step (Figs. 8A, B). In tumor cells which lack an active glycerol 3-phosphate shuttle such as MCF-7 cells, extracellular pyruvate is required to recycle NAD⁺ for the glycolytic GAPDH reaction (28). In the absence of PGMtide and exogenous pyruvate, the cells produce energy and pyruvate mainly from glutamine, thereby limiting cell proliferation (Fig. 9D). The addition of pyruvate to the medium of MCF-7 cells reactivated the flux through GAPDH, thereby increasing 3-phosphoglycerate production (Figs. 9C, 10). It can be assumed that the increased availability of 3-phosphoglycerate can boost the flow through the residual PGMtide-inhibited PGM activity in MCF-7 cells. Under these conditions energy metabolism and synthetic processes are in balance and cell proliferation is reactivated. In accordance, in the low glycolytic MDA-MB-453 cells which contain an active glycerol 3-P-shuttle, the PGMtide-induced inhibition of cell proliferation was not reversible by extracellular pyruvate (Fig. 8C).

The PGMtide and its control peptide both consist of 31 amino acids and differ in the last six amino acids: LIRHGE and RIEGHL, respectively. Therefore, it can be speculated that the specific effect of PGMtide on tumor cells was mediated by these last six amino acids. Small molecular weight compounds mimicking the PGMtide would be helpful reagents to further investigate the interaction between tumor cell proliferation and tumor cell metabolism, and may have potential as tumor therapeutic drugs.

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Footnotes

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The abbreviations used are: NDPK, nucleoside diphosphate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGM, phosphoglycerate mutase type B; NTP, nucleoside triphosphate; 2,3-BPG, 2,3-bisphosphoglycerate

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Figure legends

Fig. 1. In-situ phosphorylation screening of λ-phage cDNA expression library. A cDNA expression library (fetal brain) was plated on agar plates and the phage colonies transferred to nitrocellulose membranes previously soaked with IPTG to induce protein expression. Membranes were blocked in a buffer containing BSA and cold GTP-Mg and incubated with Nm23-H1/GAPDH complex under phosphorylation conditions. Background signals potentially resulting from NDPK autophosphorylation were reduced by incubation with excess GDP-Mg, then the membranes were subjected to autoradiography. A. Example autoradiograph from first-round screening. The phage plaque corresponding to the spot marked by an arrow was picked from the plate, eluted phages were plated again in the presence of host bacteria and the phosphorylation screening procedure repeated. B. Autoradiograph from second-round screening, using a membrane blotted with phages from A. Arrows indicate selected positive plaque spots containing cDNA inserts identified later as PGM B sequences.

Fig. 2. Phosphorylation of PGM by the Nm23-H1/GAPDH complex in the presence of $\gamma^{32}$P-GTP. A. After autoradiography of the dried SDS gel (left picture), the same gel was re-hydrated and coomassie blue-stained (right picture). Phosphorylation is dependent on the presence of the Nm23-H1/GAPDH complex (see corresponding bands on the coomassie blue-stained gel and the Nm23-H1 band on the autoradiograph) and is also observed after denaturation of PGM by heat treatment (80 °C for 2 min) (lane pair labelled “PGM heat-denat.”). B. The PGM$^{\text{His10Gly}}$ mutant is not phosphorylated by the Nm23-H1/GAPDH complex.
Fig. 3. **Time-course of PGM phosphorylation by Nm23-H1/GAPDH.** His-tagged PGM (1 µg) was phosphorylated *in vitro* in the presence of Nm23-H1/GAPDH complex (0.5 µg) and γ^{32}P-GTP for the times indicated at 30°C. Phosphorylation was stopped by snap freezing and adjusting the re-thawed solution to 8M urea. His-tagged PGM was then captured on Ni^{2+} beads, free γ^{32}P-GTP was removed by washing and the beads counted in a liquid scintillation counter.

Fig. 4. **Activation of PGM by dCTP in isoelectric focusing fractions of MCF-7 cell extract.** A. Co-focusing of PGM with NDPK and GAPDH in the pre-complex (fractions 33-37) and the glycolytic enzyme complex (38-43). The pre-complex contains Nm23-H1 (NDPK A), whereas in the glycolytic enzyme complex hybrids between Nm23-H1 and Nm23-H2 (NDPK B) are found. Pure Nm23-H2 focuses outside of both complexes in fractions 56-64. B. Activation of PGM by 2,3-BPG in the pre-complex (fractions 33-37) C. Activation of PGM by 2,3-BPG and dCTP in the pre-complex (fractions 33-37) in the presence of sodium orthovanadate.

Fig. 5. **PGMtid-induced inhibition of PGM phosphorylation by the Nm23-H1/GAPDH complex in vitro.** PGM (0.6 µg) was incubated with GAPDH/Nm23-H1 complex (0.2 µg) in the presence of different amounts of peptide as indicated at the top. Urea was added at equal concentrations (approx. 0.5 M) to all reactions to account for the urea content of the peptide solutions. Reactions were started by the addition of γ^{32}P-GTP and incubated for 10 min at 30°C. After separation of the samples by SDS-PAGE, gels were dried and exposed to film overnight.

Fig. 6. **Influence of PGMtid on activities of PGM and NDPK in vitro.** Recombinant PGM (1 µg) and GAPDH/NDPK complex (1 µg) were incubated in a reaction mixture containing
all required reagents and coupling enzymes, and PGMtide yielding the final concentrations indicated at the bottom of the figure. Reactions were started by the addition of 3-phosphoglycerate (PGM) or dTTP (NDPK) and carried out for 10 min at 30 °C. Data represent mean values (± S.D.) from three measurements, with the activities in the absence of PGMtide defined as 100%.

Fig. 7. Cellular localization of PGMtide. Fluorescein-linked peptides were added to the medium of MCF-7 cells at a final concentration of 5 µM each. After an incubation for 10 minutes at 37°C, the cells which were grown directly on glass slides were washed, fixed and counterstained with DAPI. Cells were examined by fluorescence microscopy using FITC and DAPI filters. Depicted are the overlayed microphotographs showing cytoplasmic and possibly also nuclear staining of the MCF-7 cells by fluorescein-PGMtide (A) and fluorescein-control peptide with the PGM-derived amino acids in twisted order (-RIEGHL) (B) but not by a fluorescein-PGMtide version lacking the Antennapedia homeodomain sequence (sequence: fluorescein-HHHHHHPWLIRHGE), where only DAPI-stained nuclei could be seen (C) (original magnification: x600).

Fig. 8. Influence of PGMtide on the growth of MCF-7 and MDA-MB-453 cells. Cells (1.9 x10^5) were seeded into 25 cm² flasks and PGMtide peptide or the control peptide were added to the fully supplemented culture media six hours after seeding (7 µM final concentration each). MCF-7 cells were cultured in DMEM/10% FCS in the presence of 4 mM pyruvate (A) or without extracellular pyruvate (B). At the indicated times, three flasks out of each group were removed and the cells harvested and counted. Each data point (± S.D.) represents the mean of two independent experiments with triple values each. C. Comparison of the growth inhibitory effect of PGMtide on MCF-7 (medium without pyruvate) and MDA-MB-453 cells (Leibovitz L15 medium/10% FCS containing 4 mM pyruvate) in between 24 to 48 h of
incubation. The bars show the percentage of cell growth between 24 h and 48 h of incubation with PGMtide; 100% would mean a doubling of the cell number within this time span.

Fig. 9. **Effect of PGMtide on metabolic flux rates in MCF-7 cells cultivated in the presence or absence of extracellular pyruvate.** MCF-7 cells grown in DMEM containing 10% FCS, 5 mM glucose, and 2 mM glutamine were treated with a single application of PGMtide (7 µM final concentration) in the presence or absence of 4 mM pyruvate as described under Fig. 8. At the time points indicated, cell culture supernatants were removed, centrifuged at 20,000 g and used for determinations. **A:** Correlation between serine production and glucose consumption in MCF-7 cells, cultivated in pyruvate free medium. **B:** Correlation between serine and alanine production in MCF-7 cells cultivated in pyruvate free medium. **C:** Correlation between glucose consumption and lactate production of MCF-7 cells treated with PGMtide, in medium without or with 4 mM pyruvate. **D, E:** Glutamine consumption and lactate production rates, respectively, of MCF-7 cells treated with control peptide or PGMtide, in medium without or with 4 mM pyruvate.

Fig. 10. **Interaction between glycolysis and amino acid metabolism.**

In contrast to normal proliferating cells, tumor cells, such as MCF-7 cells use glucose as well as glutamine for energy production. The increased flow of glutamine to lactate (glutaminolysis) is caused by a truncation of the citrate cycle as well as increased activities of glutaminase (E.C. 3.5.1.2) and NAD⁺ or NADP⁺-dependent malic enzyme (E.C. 1.1.1.39 and E.C. 1.1.1.40, respectively) in tumor cells (1-5). A point of interaction between glycolysis and glutaminolysis is PGM with its substrate 3-phosphoglycerate and its product 2-phosphoglycerate. 3-phosphoglycerate is the precursor for serine synthesis whereas 2-phosphoglycerate is the product of serine degradation. The amino group for serine synthesis derives from the glutaminolytic intermediate glutamate. Pyruvate as acceptor of the amino
group of serine can derive from glycolysis or glutaminolysis. It is required for alanine synthesis but also for the lactate dehydrogenase reaction which recycles NAD$^+$ required by GAPDH.

Fig. 11. **PGMtide selectively inhibits proliferation of tumor cell lines.** Different cell lines were treated with increasing amounts of the PGMtide in 12-well plates. Treatment was done by a one-time addition of PGMtide at the indicated final concentration to fresh medium, fully supplemented with FCS, glutamine and pyruvate, after the cells had settled on the plates. At the indicated day of culturing, cells were harvested without prior exchange of medium. Cells were trypsinized thoroughly and counted in a Neubauer chamber, using scans of the microphotographs and the counting function of the NIH Image public domain software (http://rsb.info.nih.gov/nih-image/). Four to six different wells were used for each data point. The legends in the diagrams indicate the concentrations of PGMtide [µM] for each curve. In all diagrams, the control curve was done in the presence of control peptide, having the PGM-derived amino acids in twisted order (-RIEGHL), at the highest concentration used for PGMtide. **A.** Small cell lung cancer cell line GLC4. **B.** Glioblastoma cell line Tx3095. **C.** Synoviocytes from rheumatoid arthritis. **D.** Amniotic fluid cells.
Table I. Inactivation of PGM by heat treatment.

PGM was heated to 80°C for the times indicated, then chilled on ice. Activity was measured after addition of substrates and coupling enzymes as described under 'Experimental procedures.' The numbers given represent mean values of the specific activity from duplicate experiments. One unit is defined as μmol 3-phosphoglycerate converted ⋅min⁻¹ at 30 °C.

| Heating time | 0     | 30 sec | 1 min | 1.5 min | 2 min | 3 min |
|--------------|-------|--------|-------|---------|-------|-------|
| PGM activity (mU/mg) | 2870  | 260    | 44    | 2       | 0     | 0     |
Fig. 2A

|                | +   | +   | +   | +   |
|----------------|-----|-----|-----|-----|
| $\gamma^{32}$P-GTP | +   | +   | +   | +   |
| Nm23/GAPDH     | +   | -   | +   | -   |
| PGM native     | +   | +   | -   | -   |
| PGM heat-denat. | -   | -   | +   | +   |

[gel image]

Autoradiograph

GAPDH →
PGl ↔
Nm23-H1 ↔

Gel

kDa

-- 46
-- 30
-- 21
Fig. 2B

B

PGM - wild type
PGM - His10Gly

← PGM
← Nm23-H1
Fig. 4A

A

[Diagram showing enzyme activity across fractions]

- ND PK
- PGM
- GAPDH

Glycolytic enzyme complex
Pre-complex

[Graph with enzyme activity plotted against fractions]
Fig. 4

B

PGM activity without vanadate

pre-complex  glycolytic enzyme complex

[U/ml]

fractions

○ without 2,3-BPG
● + 1.2mM 2,3-BPG

C

PGM activity with 0.5mM vanadate

pre-complex  glycolytic enzyme complex

[U/ml]

fractions

○ without 2,3-BPG and dCTP
△ + 0.5mM dCTP
● + 1.2mM 2,3-BPG
Fig. 5

| Peptide [µM] | 0 | 2 | 4 | 10 | 100 |
|--------------|---|---|---|----|-----|
| PGM          |   |   |   |    |     |
| Nm23-H1      |   |   |   |    |     |
| PGMtide      |   |   |   |    |     |
| Control pept.|   |   |   |    |     |

|                | PGMtide | control peptide |
|----------------|---------|-----------------|
| 0              | 0       | 0               |
| 2              | 2       | 2               |
| 4              | 4       | 4               |
| 10             | 10      | 10              |
| 100            | 100     | 100             |

![Image of gel showing PGM, Nm23-H1, PGMtide, and control peptide at different concentrations]
Fig. 6
Fig. 7

A

B

C
Fig. 8

A

MCF-7 without pyruvate

B

MCF-7 with pyruvate

| days cultivation | PGMtide | control |
|------------------|---------|---------|
| 0                | 1       | 2       |
| 0.5              | 1.5     | 2.5     |
| 1                | 2       | 3       |
| 1.5              | 2.5     | 3.5     |
| 2                | 3       | 4       |
| 2.5              | 3.5     | 4.5     |
| 3                | 4       | 5       |
| 3.5              | 4.5     | 5.5     |
Fig. 8 C

![Graph showing increase in cell number (%)](image-url)
Fig. 9

A

control peptide
slope: 0.002
intercept: 11.5
r: 0.01

PGM tide
slope: 0.19
intercept: -1.7
r: 0.819

serine production [nmol/h flask]

serine consumption [nmol/h flask]

glucose consumption [nmol/h flask]

B

PGM tide
slope: -8.56
intercept: 74
r: -0.622

control peptide
slope: 2.14
intercept: -1.8
r: 0.598

serine production [nmol/h flask]

serine production [nmol/h flask]

alanine production [nmol/h flask]

C

with 4 mM pyruvate
slope: 1.6
intercept: 23
r: 0.972

without pyruvate
slope: -0.2
intercept: 204
r: -0.460

lactate production [nmol/h flask]

glucose consumption [nmol/h flask]
Fig. 9

D  glutamine consumption
without pyruvate  with 4 mM pyruvate

|                  | control | PGMtide | control | PGMtide |
|------------------|---------|---------|---------|---------|
| [nmols/(h\times10^5 cells)] | 0       | P < 0.004 | 0       | P < 0.004 |
|                  | 25%     | 50%     | 75%     |         |

E  lactate production
without pyruvate  with 4 mM pyruvate

|                  | control | PGMtide | control | PGMtide |
|------------------|---------|---------|---------|---------|
| [nmols/(h\times10^5 cells)] | 100     | 150     | 200     | n.s.    |
|                  | P < 0.02 |         |         |         |
Fig. 11

Tumor cells

Non-tumor cells

A

B

C

D

Control
5 µM
7 µM
10 µM

Control
7 µM
14 µM

Control
7 µM
14 µM

Control
7 µM
14 µM
Phosphoglycerate mutase-derived polypeptide inhibits glycolytic flux and induces cell growth arrest in tumor cell lines
Matthias Engel, Sybille Mazurek, Erich Eigenbrodt and Cornelius Welter

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