Regulation of Cell Proliferation by Autocrine Motility Factor/Phosphoglucose Isomerase Signaling*  

Soichi Tsutsumi‡§, Takashi Yanagawa‡, Tatsuo Shimura‡§, Tomoharu Fukumori‡, Victor Hogan‡, Hiroyuki Kuwano‡, and Avraham Raz‡¶  

From the ‡Tumor Progression and Metastasis, Karmanos Cancer Institute, The Department of Pathology, Wayne State University, School of Medicine, Detroit, Michigan 48201 and §Department of General Surgical Science (Surgery I), Gunma University Graduate School of Medicine, Maebashi, 371-8511, Japan

Autocrine motility factor (AMF)/phosphoglucose isomerase (PGI; EC 5.3.1.9) is a housekeeping cytosolic enzyme that plays a key role in both glycolysis and gluconeogenesis pathways. AMF/PGI is also a multifunctional protein that displays cytokine properties, eliciting mitogenic, motogenic, and differentiation activities, and has been implicated in tumor progression and metastasis. Because little is known about AMF/PGI-dependent signaling in general and during tumorigenesis in particular, we sought to study its effect on the cell cycle. To elucidate the functional role of PGI, we stably transfected its cDNA into NIH/3T3 and BALB/c 3T3-A31 fibroblasts. Ectopic overexpression of PGI results in the acquisition of a transformed phenotype associated with an acceleration of G1 to S phase cell cycle transition. These were manifested by up-regulation of cyclin D1 expression and cyclin-dependent kinase activity and down-regulation of the cyclin-dependent kinase inhibitor p27Kip1. The reduced p27Kip1 protein expression level in PGI-overexpressing cells could be restored to control levels by treatment with proteasome inhibitor. PGI-overexpressing cells also exhibited elevated expression of Skp2 involved in p27Kip1 ubiquitination and elevation in the levels of retinoblastoma protein hyperphosphorylation. Thus, we may conclude that the overexpression of AMF/PGI enhances cell proliferation together with up-regulation of cyclin/cyclin-dependent kinase activities and down-regulation of p27Kip1, whereas the induction of 3T3 fibroblast transformation by PGI is regulated by the retinoblastoma protein pathway.

* This work was supported by National Institutes of Health Grant CA-51714 (to A. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.  
† To whom correspondence should be addressed: Tumor Progression and Metastasis, Karmanos Cancer Institute, 110 East Warren Ave., Detroit, MI 48201. Tel.: 313-833-0960; Fax: 313-831-7518; E-mail: raz@karmanos.org.  
‡ The abbreviations used are: AMF, autocrine motility factor; AMFR, AMF receptor; PGI, phosphoglucose isomerase; CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; GST, glutathione S-transferase; RT, reverse transcription; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PISK, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; SCF, Skp1-Cul1-F-box.

Received for publication, April 30, 2003, and in revised form, May 28, 2003  
Published, JBC Papers in Press, June 3, 2003, DOI 10.1074/jbc.M304537200

This paper is available on line at http://www.jbc.org
Histone H1 was obtained from Upstate Biotechnology (Lake Placid, NY), and GST-Rb 769 was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfection—A murine NIH/3T3 fibroblast cell line and its derivative cell line, BALB/c 3T3-A31 (21), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C and 5% CO2. To verify the absence of contamination by retroviral vector pcDNA3.1, pcDNA3.1, and pcDNA3.1 neo-PI were established as previously described (16) and were designated 3T3neo and 3T3PGI, respectively.

Parental A31 cells were transfected with pcDNA3.1 neo or pcDNA3.1 neo-PI using LipofectAMINE according to the manufacturer’s instructions (Invitrogen). Isolation of single clones of the stable transfectants was accomplished by adding 750 µg/ml puromycin (Invitrogen) to the culture medium. The A31 cell line, stably transfected with pcDNA3.1 neo or pcDNA3.1 neo-PI, was designated A31neo or A31PI, respectively. All experiments were repeated at least three times, and results were confirmed using both clonal cell lines and the pooled cell population.

Cell Proliferation Assay—Cell proliferation assays were performed by seeding cells at a density of 1 × 104 cells/well in 6-well plates. Cells were fed DMEM with 10% FBS every other day, and the number of cells were manually counted with a hemocytometer.

Cell Cycle Synchronization and DNA Content Analysis—Cell cycle phase distribution was determined by flow cytometry of propidium iodide-stained cells. Whole cell suspensions were washed in phosphate-buffered saline (PBS), fixed in 70% ethanol, stained in 50 µg/ml propidium iodide, 1 mg/ml RNase, 0.1% Triton X-100, and analyzed with a BD Biosciences.

For cell synchronization, exponentially growing cells were treated for 18 h with 100 µg/ml nocodazole to induce G2-M arrest (22). Mitotic cells were collected by gentle pipetting and were reseeded into fresh DMEM with 10% FBS. At various times after plating, cells were collected and their cell cycle distribution was determined by flow cytometry as described above.

Western Blot Analysis—The cells were lysed in lysis buffer (50 mM HEPES, pH 7.9, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 1% sodium deoxycholate, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM β-glycerophosphate, and 0.1 mg/ml leupeptin) at 4 °C. Cell lysates containing equal amounts of protein were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (MSI, Westborough, MA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T) incubated with primary antibody for 2 h, washed 3 times for 15 min in TBS-T, incubated with the secondary horseradish peroxidase-conjugated antibody (Zymed Laboratories Inc., San Francisco, CA) for 1 h, and finally washed three times. The horseradish peroxidase activity was detected by an incubation of the membrane with enhanced chemiluminescence reagent (Amer sham Biosciences). A Kodak imaging system determined the intensity of bands.

RNA Isolation and Reverse Transcription (RT)-PCR—Total cellular RNA was isolated according to the manufacturer’s instructions. Using TRIZol reagent (Invitrogen). RT-PCR analysis was performed as described (23). For cyclin D1 cDNA, 20 amplification cycles (93 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) were performed with the following primers: mouse cyclin D1, 5′-TCGACACCAATCTTCAACGAC-3′ (forward) and 5′-GGCCGCGTCTTCTTCTACGAGC-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5′-CATGCGGTAATCATGCTGCACCAGA-3′ (forward) and 5′-GGTCCACACCTGGTCTGATGCCCG-3′ (reverse). In all cases, PCR reagents were shown to be in a linear range by performing parallel control PCR with increasing template cDNA concentration. The absence of contamination of RNA samples with DNA, we performed the PCR on samples that were processed identically to the target samples but were not reverse-transcribed. After RT-PCR, samples were electrophoresed on a 1.5% agarose gel containing 10 µg/ml ethidium bromide, and the intensity of the bands was measured by a Kodak Digital Science Image System 44C.

Immunofluorescence Microscopy—Cells were seeded on coverslips in 6-well plates at a density of 1 × 104 cells/well in DMEM containing 10% FBS. The next day, the medium was changed to fresh DMEM with or without 10% FBS. After 36 h incubation, cells were fixed in 4% paraformaldehyde/PBS for 10 min and permeabilized with 0.1% Triton X-100/PBS for 15 min. The cells were blocked with 1% bovine serum albumin in PBS for 30 min at 4 °C and then incubated with anti-p27Kip1 antibodies (1:100 dilution in 0.1% bovine serum albumin in PBS) at 4 °C for 2 h. After 3 washes with 0.05% Triton X-100/PBS, cells were incubated with 1 µg/ml fluorescein isothiocyanate-conjugated anti-rabbit IgG diluted 1:200 in 0.05% Triton X-100/PBS. After three washes with PBS, coverslips were then mounted on a glass slide with a drop of SlowFade reagent (Molecular Probes, Eugene, OR). Immunofluorescence was recorded with a Sony digital CCD camera (DXC-970MD) mounted on an Olympus BX40 microscope.

In Vitro CDR Assay—CDR2 and CDR4 kinase assays were performed as described to assay proteasome targeting of p27Kip1. Cells lysates prepared after each incubation period were analyzed by Western blotting with p27Kip1 antibody as described above.

Immunofluorescence Microscopy—Cells were seeded on coverslips in 6-well plates at a density of 1 × 104 cells/well in DMEM containing 10% FBS. The next day, the medium was changed to fresh DMEM with or without 10% FBS. After 36 h incubation, cells were fixed in 4% paraformaldehyde/PBS for 10 min and permeabilized with 0.1% Triton X-100/PBS for 15 min. The cells were blocked with 1% bovine serum albumin in PBS for 30 min at 4 °C and then incubated with anti-p27Kip1 antibodies (1:100 dilution in 0.1% bovine serum albumin in PBS) at 4 °C for 2 h. After 3 washes with 0.05% Triton X-100/PBS, cells were incubated with 1 µg/ml fluorescein isothiocyanate-conjugated anti-rabbit IgG diluted 1:200 in 0.05% Triton X-100/PBS. After three washes with PBS, coverslips were then mounted on a glass slide with a drop of SlowFade reagent (Molecular Probes, Eugene, OR). Immunofluorescence was recorded with a Sony digital CCD camera (DXC-970MD) mounted on an Olympus BX40 microscope.

In Vitro CDR Assay—CDR2 and CDR4 kinase assays were performed as described to assay proteasome targeting of p27Kip1.
formed as described previously (18). Briefly, 500 
μg of protein extracts were immunoprecipitated with 2 
μg of the anti-CDK2 or anti-CDK4 antibodies for 60 min at 4
° C. Immunoprecipitated proteins were col-
lected on protein A-Sepharose® 6MB (Amersham Biosciences). Kinase
reactions were performed for 30 min at 30
° C in kinase assay buffer (50
mM HEPES, pH 7.2, 10 mM MgCl₂, 2.5 mM EGTA, 0.1
mM NaF, and 0.1 
mM Na₃VO₄) and contained 20 
μM [γ-³²P]ATP at a specific activity of 10
Ci/mmol and 1 
μg of histone H1 for CDK2 assays or 1 
μg of GST-Rb 769 
for CDK4 assays reactions. Reaction products were resolved by SDS-
PAGE. The gels were stained in Coomassie Blue, dried, and exposed to
film. A Kodak imaging system determined the density of the bands.
Normal rabbit immunoglobulin G (Sigma) was used as a negative
control in immunoprecipitation experiments.

Statistical Analysis—Associations between the variables were tested 
by Student’s t test or Fisher’s exact test. All statistical differences were
deemed significant at the level of p < 0.05.

RESULTS

PGI Shortens the G₁ Interval—We have established two dif-
ferent types of murine fibroblast based cell lines (NIH/3T3 and
A31) stably transfected and overexpressing AMF/PGI. Three
clones exhibiting high level expression of PGI in both NIH/3T3
and A31 cells were selected. The ratios of PGI expression 
of each clone were 5 to 6 compared with the empty vector-transfected 
control (Fig. 1A). PGI secretion was restricted to the 
PGI-transfected cells and could not be detected in the conditioned 
medium of the parental and empty vector-transfected 
cells (Fig. 1A). All AMF/PGI-overexpressing clones grew ~2-
fold faster than those of parental or empty vector-transfected 
control cells (Fig. 1A). The effect of ectopic expression of PGI on 
cell cycle distribution was determined next. There was no spe-
cific accumulation of the PGI-overexpressing cells at any cell 
cycle phase relative to the control cells (Fig. 1C). The percent-
age of G₁ phase cells was slightly lower, and the proportion of 
the S phase cells was respectively higher in the PGI-overex-
pressing cells, but this did not change significantly. To study 
the effect of PGI on cell cycle progression, the length of G₁ 
phase was determined. Cells were treated with the mitotic 
inhibitor nocodazole for 18 h followed by shaking and replating 
in nocodazole-free medium. The cells were collected at the 
indicate time points after the mitotic shake, and the DNA 
profiles are shown in Fig. 2. At 8 h after replating, PGI-over-
expressing cells shifted toward S phase (increased DNA con-
tent) as compared with parental and empty vector-transfected 
control cells (Fig. 2A, bottom panel, arrow). The presence of S
phase in control cells could not be detected prior to 10 h after 
cells were replated (top and second panels). Similar results 
were obtained from two other PGI-overexpressing clones and A31 and its derivative cells. y axis, cell number; x axis, DNA content. B, the table shows the percentage of G₁/G₀, S, G₂/M populations of cell at 8 h after replating. PGI-overexpressing cells showed a shift toward S phase. Data represent the mean of triplicate experiments ± S.D. *, significant difference when compared with control cell (p < 0.01).

**Fig. 2.** PGI overexpression results in a shortened G₁ phase. Mitotically arrested cells were isolated using nocodazole and replated. Cells 
were collected at indicated times, and DNA content was analyzed by flow cytometry. Similar results were obtained in three independent 
experiments (A). Top panel, parental NIH/3T3. Second panel, empty vector-transfected NIH/3T3. Bottom panel, PGI-overexpressing NIH/3T3 clone 
1. Similar results were obtained from other PGI-overexpressing clones and A31 and its derivative cells. y axis, cell number; x axis, DNA content. 
B, the table shows the percentage of G₁/G₀, S, G₂/M populations of cell at 8 h after replating. PGI-overexpressing cells showed a shift toward S 
phase. Data represent the mean of triplicate experiments ± S.D. *, significant difference when compared with control cell (p < 0.01).

**Autocrine Motility Factor Target Cell Cycles**

| Cell lines | G₁/G₀(%) | S(%) | G₂/M(%) |
|------------|---------|------|--------|
| NIH/3T3    | 62.7 ± 2.2 | 5.2 ± 0.4 | 32.1 ± 2.0 |
| 3T3zco     | 64.4 ± 3.4 | 4.7 ± 0.3 | 30.8 ± 3.3 |
| 3T3PGI1    | 50.8 ± 1.5 | 18.3 ± 0.9* | 30.9 ± 2.4 |
| 3T3PGI2    | 48.4 ± 2.9 | 20.3 ± 1.0* | 31.2 ± 2.1 |
| 3T3PGI3    | 50.9 ± 2.1 | 18.8 ± 1.3* | 30.3 ± 1.5 |
| A31        | 63.0 ± 0.9 | 4.2 ± 0.3 | 32.8 ± 1.1 |
| A31zco     | 61.3 ± 1.5 | 3.9 ± 1.0 | 34.8 ± 0.9 |
| A31PGI1    | 51.2 ± 1.8 | 13.9 ± 0.7* | 34.9 ± 1.4 |
| A31PGI2    | 49.7 ± 1.0 | 15.3 ± 0.8* | 35.0 ± 1.5 |
| A31PGI3    | 51.0 ± 3.0 | 13.3 ± 0.6* | 34.3 ± 1.3 |

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
overexpression of NIH/3T3 and A31 cell clones as compared with
the respective control cells (Fig. 3A). The levels of cyclin A,
cyclin E, and p53 remained essentially unchanged in the PGI-
overexpressing cells as compared with the controlled cells. For
a more sensitive analysis, we performed RT-PCR analyses of
the expression levels of cyclin D1 and confirmed that cyclin D1
mRNA was increased 3-fold in the PGI-overexpressing cells as
compared with their normal counterparts (Fig. 3B). Furthermore,
in contrast to p21Cip1, the level of p27Kip1 was decreased
more, in contrast to p21Cip1, the level of p27Kip1 was decreased
by ~50–60% relative to the control cells. The above-mentioned
pattern of the cell cycle components change in expression in the
PGI-overexpressing cells was the same, at 2 and 3 days of
culture.

PGI Promotes Proteasome-dependent Degradation of p27Kip1
Protein—Because of the fact that p27Kip1 increases in serum-
starved and contact-inhibited normal cells (24), we questioned
the status of p27Kip1 in PGI-transfected cells under diverse
culture conditions. Cells were either grown to confluence or
cultured as serum-starved for 24 h at 37 °C. P27Kip1 expression
analysis revealed that in PGI-overexpressing cells it was sig-
ificantly reduced (60–70%) compared with the cells both in
density-arrested and in serum-starved conditions (Fig. 4A). Next
we examined whether the reduced p27Kip1 protein level
was due to change in protein stability by exposing cells to
cycloheximide treatment. P27Kip1 protein was rapidly degraded
(80–90% reduced) in cycloheximide-treated PGI-overexpress-
ing NIH/3T3 and A31 cells, whereas it was more stable in
control cells at 4 and 8 h (Fig. 4B). Altered regulation of p27Kip1
protein stability was probably the major cause of its reduced
protein expression in PGI (overexpressing) cells. Thus, we
questioned whether p27Kip1 degradation processed was dis-

Fig. 3. Expression of cell cycle-related proteins in PGI-over-
expressing NIH/3T3 and A31 cells. A, cell cycle-regulated proteins
were analyzed by immunoblotting. Up-regulation of cyclin D1 and
down-regulation of p27Kip1 were observed in PGI-overexpressing NIH/
3T3 and A31 cells. Equal loading was confirmed by immunoblotting the
membrane with an antibody to actin (bottom row). B, RT-PCR analysis
of cyclin D1 mRNA levels using glyceraldehyde-3-phosphate dehydro-
genase (GAPDH; bottom row) as the internal control. PCR products
were electrophoresed through 1.5% agarose gel and stained with
ethidium bromide. First lane, parental NIH/3T3; second lane, empty
vector-transfected NIH/3T3; third through fifth lanes, PGI-overexpress-
ing NIH/3T3 clone 1, 2, and 3, respectively; sixth lane, parental A31;
seventh lane, empty vector-transfected A31; eighth through tenth lanes,
PGI-overexpressing A31 clone 1, 2, and 3, respectively.

Fig. 4. PGI overexpression induces p27Kip1 down-regulation in
NIH/3T3 and A31 fibroblasts. The amount of p27Kip1 is decreased in
the PGI-overexpressing NIH/3T3 and A31 cells. A, upper row, cells were
grown to near confluence (95–100%). Lower row, cells were cultured in
serum starvation conditions for 24 h. First lane, parental NIH/3T3;
second lane, empty vector-transfected NIH/3T3; third through fifth,
PGI-overexpressing NIH/3T3 clone 1, 2, and 3, respectively; sixth lane,
parental A31; seventh lane, empty vector-transfected A31; eighth
through tenth lanes, PGI-overexpressing A31 clone 1, 2, and 3,
respectively. B, cells were treated with the protein synthesis translational
inhibitor cycloheximide to compare the stability of p27Kip1 protein.
Total cellular proteins were resolved on SDS-PAGE and immunoblotted
with specific antibodies to p27Kip1. Similar results were obtained from
other PGI-overexpressing clones. C, cells were treated for 12 h with the
proteasome inhibitor lactacystin. Western blot analysis of p27Kip1
expression was then performed. Similar results were obtained from
other PGI-overexpressing clones. D, upper row, expression of Skp2 is up-
regulated in PGI-overexpressing cells determined by Western blotting;
lower row, up-regulation of Skp2 expression was inhibited by anti-PGI
IgG. First lane, parental NIH/3T3; second lane, empty vector-trans-
fected NIH/3T3; third through fifth lanes, PGI-overexpressing NIH/3T3
clone 1, 2, and 3, respectively; sixth lane, parental A31; seventh lane,
empty vector-transfected A31; eighth through tenth lanes, PGI-over-
expressing A31 clone 1, 2, and 3, respectively.
rupted by the 26 S protease system inhibitor in PGI-overexpressing cells. To address this, we used the 26 S protease-specific inhibitor lactacystin and found that lactacystin treatment restores p27\textsuperscript{Kip1} expression level in PGI-overexpressing cells and to that of the control cells (Fig. 4C). The F-box protein is the substrate-specific recognition component of Skp1-Cul1-F-box (SCF) ubiquitin-protein ligase complexes that is used to target specific proteins for degradation (25). Thus, we tested whether PGI alters the expression of Skp2, a member of the F-box family, leading to an increased ubiquitination and subsequent degradation of p27\textsuperscript{Kip1} protein. As shown in Fig. 4D, Skp2 protein level was slightly elevated in PGI-overexpressing cells. The degradation of p27\textsuperscript{Kip1} was partially impaired by the anti-PGI antibody treatment, suggesting that PGI-signaling inhibition might impact the degradation process of p27\textsuperscript{Kip1} (data not shown). Up-regulation of Skp2 expression was inhibited by anti-PGI IgG (Fig. 4D).

Next, the subcellular localization of p27\textsuperscript{Kip1} was determined by anti-p27\textsuperscript{Kip1} indirect immunofluorescent staining. No differences in the distribution of p27\textsuperscript{Kip1} localization were detected

![Subcellular localization of p27\textsuperscript{Kip1} in PGI-overexpressing cells.](image_url)

**FIG. 5.** Subcellular localization of p27\textsuperscript{Kip1} in PGI-overexpressing cells. Cells were serum-starved for 36 h, fixed in 4% paraformaldehyde, and incubated with anti-p27\textsuperscript{Kip1} antibody. In asynchronously growing parental, empty vector-transfected, and PGI-overexpressing NIH/3T3 and A31 cells, p27\textsuperscript{Kip1} was detected in the nucleus and cytoplasm (data not shown). p27\textsuperscript{Kip1} was expressed mainly in the nucleus during serum starvation in parental and empty vector-transfected cells. In contrast, no accumulation of p27\textsuperscript{Kip1} was observed in the nucleus of PGI-overexpressing cells during serum starvation. a, parental NIH/3T3; b, empty vector-transfected NIH/3T3; c, PGI-overexpressing NIH/3T3 clone 1; d, parental A31; e, empty vector-transfected A31; f, PGI-overexpressing cells A31 clone 1. Similar results were obtained from other PGI-overexpressing clones.

![CDK activities and phosphorylation of the Rb protein.](image_url)

**FIG. 6.** CDK activities and phosphorylation of the Rb protein. A, expression of CDK2 and CDK4 protein in PGI-overexpressing cells. Equalized protein samples were subjected to Western blot analysis with antibodies to CDK2 and CDK4. No differences in expression of CDK2 and CDK4 were observed between PGI-overexpressing and control cells. The bottom row was probed with anti-actin antibody as a control. B, total CDK2 and CDK4 activities in the PGI-overexpressing NIH/3T3 and A31 versus control cells. Equal amounts (0.5 mg) of total proteins from the cell lysates were immunoprecipitated with 2 \( \mu \)g of anti-CDK2 and anti-CDK4 antibodies. The in vitro immunocomplex kinase assays were performed with histone H1 or GST-Rb fusion protein as the substrate, as described under “Experimental Procedures.” The reaction products were resolved on SDS-PAGE and exposed to x-ray film. C, the phosphorylation status of Rb in the PGI-overexpressing and control cells. First lane, parental NIH/3T3; second lane, empty vector-transfected NIH/3T3; third through fifth lanes, PGI-overexpressing NIH/3T3 clone 1, 2, and 3, respectively; sixth lane, parental A31; seventh lane, empty vector-transfected A31; eight through tenth lanes, PGI-overexpressing A31 clone 1, 2, and 3, respectively.
Alterations of CDK2 and CDK4 Activity in PGI Overexpressing Cells—To continue the analysis we tested the level of CDK2 and CDK4 protein expression and found no differences between PGI-overexpressing and control cells (Fig. 6A). Next, we analyzed the CDK activities in both PGI-overexpressing and control cells. Cell lysates were immunoprecipitated with anti-CDK2 and anti-CDK4 antibodies, and the kinase activities of the immunocomplexes were determined with [γ-32P]ATP and histone H1 or GST-Rb fusion protein as a substrate. Total kinase activity of CDK2 and CDK4 was elevated in each PGI-overexpressing NIH/3T3 and A31 clones compared with control cells (Fig. 6B). The ratios of CDK activity, determined by densitometry, were 1.9–2.8 (CDK2) and 2.1–2.7 (CDK4), respectively, as compared with control cells. These results imply that PGI overexpression leads to up-regulation not only of cyclin D1 expression level but also of its associated kinase activities.

Cyclin D1/Rb/E2F Pathway Activated in PGI-transfected Cells—The Rb pathway is essential for the formation of numerous tumors (20), and hypophosphorylated Rb binds to a subset of E2F complexes, converting them to repressors that constrain expression of E2F target genes (26). Phosphorylation of Rb frees these E2Fs, enabling them to transactivate the same genes, a process initially triggered by the CDKs and then accelerated by the cyclin E-CDK2 complex (20). Thus, we questioned the status of Rb in PGI-overexpressing cells. The cells displayed an increase in the hyperphosphorylated form of Rb, which migrated in the gel more slowly than the hypophosphorylated form when compared with the parental and empty vector-transfected cells (Fig. 6C). This suggests that the Rb plays a role in the transformation induction in PGI overexpression cells.

DISCUSSION

PGI is a ubiquitous cytosolic enzyme that plays a key role in both glycolysis and gluconeogenesis pathways (1) and, therefore, a housekeeping gene transcribed in all cells. PGI is up-regulated in a variety of human cancer cells (11–13) activated by ras (5) and myc (27) and, as a result, acts as a transforming agent (16). We examined the cell cycle-associated activities related to PGI overexpression in fibroblasts. The results presented here and summarized in Fig. 7 suggest that AMF/PGI is involved in the transition from G1 to S phase during cell cycle progression, which might explain its role(s) in the modulation of tumor growth. Initially, PGI up-regulates cyclin D1 mRNA and protein expression levels. Of note, cyclin D1 is frequently overexpressed in human cancers, such as parathyroid adenoma, lymphoma, and breast cancer (28), and overexpression of cyclin D1 leads to a shortened duration of the G1 phase and reduces serum dependence in fibroblasts (18, 29–31). The activity of CDK4 was also found to be elevated in both the PGI-overexpressing NIH/3T3 and A31 cells. A similar up-regulation of CDK4 activity has been observed in NIH/3T3 cells by transformed ras (32) and myc (33), suggesting that an increase in cyclin D1-CDK4 activity may be a relatively common event in cellular transformation. Cyclin D1 promoter activity and mRNA levels are induced by various growth factors, including epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor, keratinocyte growth factor, and insulin-like growth factor-1 (34–40). These growth factors and their receptors activate Ras family members to mediate a signal transduction cascade of successive phosphorylation steps, leading to the activation of mitogen-activated protein kinase (41). It has been demonstrated that the Ras-mitogen-activated protein kinase pathway plays a pivotal role in cyclin D1 synthesis and assembly with CDK4, and nuclear retention of the enzymes depends on Ras/mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K)/Akt signaling (42). Glycogen synthase kinase-3β can phosphorylate cyclin D1 to trigger its nuclear export and pro teaseal degradation (19). PI3K and Akt kinase negatively regulate glycogen synthase kinase-3β to enhance the stability of the cyclin D-dependent kinase (19).

Previously, we have shown that ectopic expression of PGI induced activation of the PI3K/Akt-signaling pathway (16). Indeed, the PI3K inhibitor LY294002 nearly abolishes up-regulation of cyclin D1 protein levels induced by PGI overexpression in NIH/3T3 and A31 cells (data not shown). Thus, it may be concluded that up-regulation of cyclin D1 could be attributed in part to the PGI activation of the PI3K/Akt pathway. In PGI-overexpressing cells p27Kip1 levels are decreased,
p27Kip1 inhibits cyclin E-CDK2 activity, and export of p27Kip1 to the cytoplasm is usually a prerequisite for its degradation. Relegated to the cytoplasm, p27Kip1 is unable to control CDKs target of the cyclin D- and E-dependent kinases. In this study, we found an increase in the phosphorylation of Rb in PGI-overexpressing cells. Nevertheless overexpression of PGI may affect the cell cycle in multiple ways, all of which may contribute to transformation. PGI may transform fibroblasts through the CDK/Rb/E2F pathway. Now, it is pertinent to identify additional targets and examine their role in PGI regulation since the PGI-signaling pathway(s) may represent novel targets for cancer therapy.

REFERENCES

1. Harrison, R. A. (1974) Anal. Biochem. 61, 509–507
2. Watanabe, H., Carmi, P., Hogan, V., Raz, T., Silletti, S., Nabi, I. R., and Raz, A. (1991) J. Biol. Chem. 266, 3444–3448
3. Watanabe, H., Takehashi, D., Date, M., Shinozaki, T., and Raz, A. (1996) Cancer Res. 56, 2960–2963
4. Chaput, M., Claes, V., Portetelle, D., Cludts, I., Cravador, A., Burny, A., Gras, H., and Tartar, H. (1998) Nature 332, 454–455
5. Liotta, L. A., Mandel, R., Murano, G., Katz, D. A., Gordon, R. K., Chiang, P. K., and Schiffman, E. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3302–3306
6. Nabi, I. R., Watanabe, H., and Raz, A. (1992) Cancer Metastasis Rev. 11, 5–20
7. Shimizu, K., Tani, M., Watanabe, H., Nagamachi, Y., Ninakaya, Y., Shiroshi, T., Ohwada, S., Raz, A., and Yokota, J. (1999) FEBS Lett. 455, 290–300
8. Ninakaya, Y., Paku, S., Haga, A., Watanabe, H., and Raz, A. (1998) Cancer Res. 58, 2667–2674
9. Baumann, M., Kappl, A., Lang, T., Brand, K., Siegfried, W., and Paterok, E. (1999) Cancer Invest. 17, 35–36
10. Guirgis, I., Javadpour, N., Sharar, S., Biewa, c.-e., Alamin, W., Mansur, I., and Kim, J. S. (1990) J. Occup. Med. 32, 846–853
11. Nakamura, S., Watanabe, M., Imamura, S., Furukawa, H., Ishikawa, O., Sasaki, Y., Kabuto, T., and Raz, A. (1994) Cancer 74, 1855–1862
12. Maruyama, K., Watanabe, H., Shiroshi, H., Takayasu, T., Gofuka, J., Yano, Y., Inoue, M., Tamura, S., Raz, A., and Monden, M. (1995) Int. J. Cancer 64, 316–321
13. Takanami, I., Takeuchi, K., Naruke, M., Kosaira, S., Tanaka, F., Watanabe, H., and Raz, A. (1994) Cancer Res. 54, 384–389
14. Funasaka, T., Haga, A., Raz, A., and Nagase, H. (2001) Int. J. Cancer 101, 217–223
15. Shigeto, S., and Raz, A. (1993) Biochem. Biophys. Res. Commun. 194, 446–457
16. Tsutsu, S., Hogan, V., Nabi, I. R., and Raz, A. (2003) Cancer Res. 63, 242–249
17. Sherry, C. J. (2000) Cancer Res. 60, 3689–3695
18. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J., and Kay, J. M. (2003) Mol. Cell. Biol. 23, 1501–1512
19. Sherry, C. J., and Roberts, J. M. (1998) Genes Des. 13, 1501–1512
20. Sherry, C. J., and McCormick, F. (2002) Cancer Cell 2, 103–112
21. Zibib, I., and Raz, A. (1985) Int. J. Cancer 36, 261–272
22. Jordan, M. A., Thrower, D., and Wilson, L. (1992) J. Cell Sci. 101, 367–369
23. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massagué, J. (1994) Cell 78, 59–66
24. Nakayama, K. I., Hatakeyama, S., and Nakayama, K. (2001) Biochem. Biophys. Res. Commun. 282, 853–860
25. Weintraub, S. J., Prater, C. A., and Dean, D. C. (1992) Nature 358, 259–261
26. Oshur, C. R., Shin, H., Kim, S., Li, Q., Reddy, R., Mukherjee, B., Xu, Y., Winsley, D., Lee, A. I., and Dang, C. V. (2000) J. Biol. Chem. 275, 21793–21790
27. Sherr, C. J. (1996) Science 274, 1672–1677
28. Jiang, W., Kahn, S. M., Zhang, Y. J., Cacace, A. M., Infante, A. S., Doi, S., and Reed, S. I. (1994) Mol. Cell. Biol. 14, 7506–7516
29. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M., and Sherr, C. J. (1994) Cancer Res. 54, 3302–3306
30. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M., and Sherr, C. J. (1994) Oncogene 8, 3447–3457
31. Resnitzky, D., Gossen, M., Bujard, H., and Reed, S. I. (1995) Mol. Cell. Biol. 14, 1669–1679
32. Peiper, D. S., Upton, T. M., Madha, M. H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J. A., and Ewen, M. R. (1997) Nature 386, 177–181
33. Maller, M. K., Obaya, A. J., and Sedyi, J. M. (1999) Mol. Cell. Biol. 19, 4672–4683
34. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, D., and Pestell, R. G. (1995) J. Biol. Chem. 270, 23589–23597
35. Lin, S. Y., Makino, K., Xia, W., Matin, A. A., Winn, E. Y., Kwong, K. Y., Bourguignon, L., and Hung, M. C. (2001) Nat. Cell Biol. 3, 802–808
36. Page, K. L., Li, J., and Hershenson, M. B. (1999) Am. J. Respir. Cell Mol. Biol. 20, 1384–1392
37. Resnitzky, D., Gossen, M., Bujard, H., and Reed, S. I. (1995) Mol. Cell. Biol. 14, 1669–1679
38. Reffel, C. J., Sheehan, S. M., Taylor, R. G., Kendall, T. L., and Rice, G. M. (1995) Cell Physiol. 105, 307–312
39. Holthofer, W., Füller, K., Wolf, M., Koff, A., Ashin, A. W., Albanese, C., Neumeister, P., Pestell, R. G., and Petzelbauer, P. (2002) J. Biol. Chem. 277, 45847–45853
40. Li, J., J., and L., H., and Wilson, S. E. (2001) Curr. Eye Res. 25, 69–76
41. Harmelers, I. H., van Schaik, R. F., Sipkema, J., Sussenbach, J. S., and Steenbergh, P. H. (2002) J. Biol. Chem. 277, 47645–47652
42. Weinstein-Oppenheimer, C. R., Blalock, W. L., Steelman, L. S., Chang, F., and McBrey, J. A. (2000) Pharmacol. Ther. 88, 229–279
43. Pruitt, K., and Der, C. J. (2001) Cancer Lett. 171, 1–10
43. Aktas, H., Cai, H., and Cooper, G. M. (1997) Mol. Cell. Biol. 17, 3850–3857
44. Muller, D., Bouchard, C., Rudolph, B., Steiner, P., Stuckmann, I., Saffrich, R., Ansorge, W., Huttner, W., and Eilers, M. (1997) Oncogene 15, 2561–2576
45. Leone, G., DeGregori, J., Sears, R., Jakoi, L., and Nevins, J. R. (1997) Nature 387, 422–426
46. Johnson, D., Frame, M. C., and Wyke, J. A. (1998) Oncogene 16, 2017–2028
47. Lloyd, R. V., Erickson, L. A., Jin, L., Kuhl, K., Qian, X., Cheville, J. C., and Scherihauer, B. W. (1999) Am. J. Pathol. 154, 313–323
48. Tsihlias, J., Kapusta, L. R., and Slingerland, J. (1999) Annu. Rev. Med. 50, 401–423
49. Tomoda, K., Kubota, Y., and Kato, J. (1999) Nature 398, 160–165
50. Blain, S. W., and Massague, J. (2002) Nat. Med. 8, 1076–1078
51. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. (1995) Science 269, 682–685
52. Carrano, A. C., Kytan, E., Hershko, A., and Pagano, M. (1999) Nat. Cell Biol. 1, 193–199
53. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995) Cell 82, 915–925
54. Gstaiger, M., Jordan, R., Lim, M., Catzavelos, C., Mestan, J., Slingerland, J., and Krek, W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5043–5048
55. Kudo, Y., Kitajima, S., Sato, S., Miyauchi, M., Ogawa, I., and Takata, T. (2001) Cancer Res. 61, 7044–7047
56. Hershko, D., Bornstein, G., Ben-Izhak, O., Carrano, A., Pagano, M., Krausz, M. M., and Hershko, A. (2001) Cancer 91, 1745–1751
57. Mamillapalli, R., Gavršlova, N., Mihaylova, V. T., Tvetkov, L. M., Wu, H., Zhang, H., and Sun, H. (2001) Curr. Biol. 11, 263–267
58. Cantley, L. C., and Neel, B. G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4240–4245
59. Fang, S., Lorick, K. L., Jensen, J. P., and Weissman, A. M. (2003) Semin. Cancer Biol. 13, 5–14
60. Dowesward, J. (2003) Nat. Rev. Cancer 3, 11–22
61. Nobes, C. D., and Hall, A. (1995) Cell 81, 53–62
62. Weber, J. D., Hu, W., Jefcoat, S. C., Raben, D. M., and Baldassare, J. J. (1997) J. Biol. Chem. 272, 32966–32971
63. Westwick, J. K., Lambert, Q. T., Clark, G. J., Symons, M., Van Aelst, L., Pestell, R. G., and Der, C. J. (1997) Mol. Cell. Biol. 17, 1324–1335
64. Gjoerup, O., Lukas, J., Bartek, J., and Willumsen, B. M. (1998) J. Biol. Chem. 273, 18812–18818
65. Tsutsumi, S., Gupta, S. K., Hogan, V., Collard, J. G., and Rax, A. (2002) Cancer Res. 62, 4484–4490
Regulation of Cell Proliferation by Autocrine Motility Factor/Phosphoglucone Isomerase Signaling
Soichi Tsutsumi, Takashi Yanagawa, Tatsuo Shimura, Tomoharu Fukumori, Victor Hogan, Hiroyuki Kuwano and Avraham Raz

J. Biol. Chem. 2003, 278:32165-32172. doi: 10.1074/jbc.M304537200 originally published online June 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304537200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 65 references, 26 of which can be accessed free at http://www.jbc.org/content/278/34/32165.full.html#ref-list-1