Overexpression of the Autocrine Motility Factor/Phosphoglucose Isomerase Induces Transformation and Survival of NIH-3T3 Fibroblasts

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ABSTRACT

Autocrine motility factor (AMF)/phosphoglucose isomerase (PGI) is a ubiquitous cytosolic enzyme and is produced as a leaderless secretory protein, released from cells via a nonclassical pathway. AMF/PGI acts extracellularly as a potent mitogen/cytokine (CXXC, chemokine). Increased expression of AMF/PGI and its receptor/CXXC-R has been found in a wide spectrum of malignancies, and is associated with cancer progression and metastasis. To directly elucidate the functional role of AMF/PGI on cell motility and neoplastic transformation, we stably transfected AMF/PGI cDNA into NIH-3T3 cells. Ectopic overexpression of AMF/PGI results in its secretion and activation via a constitutive autocrine activation loop that renders the cells highly motile, acquiring a transformed phenotype in vitro and tumorigenicity in vivo. The transformed phenotype of AMF/PGI-transfected cells leads in part resistance to induction of apoptosis induced by serum starvation, through the activation of phosphatidylinositol 3'-kinase/Akt signaling pathway and down-regulation of caveolin-1 expression. Overexpression of this housekeeping gene induces resistance to apoptosis and neoplastic transformation, and, thus, AMF/PGI represents a novel class of oncopgenic protein.

INTRODUCTION

PGI (EC 5.3.1.19) is a ubiquitous cytosolic enzyme that plays a key role in both glycolysis and gluconeogenesis pathways (1). On secretion PGI acts as a potent mitogen/cytokine i.e., tumor AMF, neuroleukin, and maturation factor (2–4), and therefore represents a unique example of a "moonlighting protein" that exhibits multiple cellular functions (5). PGI/AMF/neuroleukin/MF is an orphan CXXC chemo
ty factor; AMFR, autocrine motility factor receptor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; PI3K, phosphatidylinositol 3'-kinase; MAPK, mitogen-activated protein kinase; PKB, protein kinase B.

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The abbreviations used are: PGI, phosphoglucose isomerase; AMF, autocrine motility factor; AMFR, autocrine motility factor receptor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; PI3K, phosphatidylinositol 3’-kinase; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; PKB, protein kinase B.

only stimulates tumor cell motility in an autocrine manner, but that it also acts as a paracrine factor on normal cells. Therefore, the AMF/PGI/neuroleukin/MF cytokine exhibits multifunctional growth factor-like activity.

A number of growth factors, such as IGF-I, hepatocyte growth factor, PDGF, FGF, and nerve growth factor have been reported to induce cell transformation and to promote cell survival (22–26). Both cellular proliferation as well as tumor cell survival are crucial for malignant progression. It was shown that preventing programmed cell death by tumor cells frequently evolve the expression of secreted growth factors that induce survival signaling pathways. Here, we show that overexpression of AMF/PGI in NIH-3T3 fibroblasts results in its secretion and the induction, via autocrine activation, of enhanced cell proliferation, transformation, and tumorigenicity in nude mice. Caveolae are vesicular invaginations of the plasma membrane that participate in cellular transport processes and signal transduction-related events (27). Caveolin is a principal component of caveolae membranes in vivo (28). Caveolin-1 is down-regulated or absent in oncogene (v-abl, hcr-abl, Ha-ras)-transformed NIH 3T3 cells (29), as well as in human cancer cells (30). Transformation of NIH-3T3 cells by AMF/PGI leads to reductions in cellular levels of caveolin and caveolin-1. More particularly, forced expression of AMF/PGI offers significant protection from apoptosis mediated by PI3K/Akt signaling pathways. Overexpression and secretion of this housekeeping gene is a novel and specific determinant of cell survival that contributes to tumor progression.

MATERIALS AND METHODS

Materials. LY294002, PI3K inhibitor, and PD98059, MAPK kinase inhibitor were from Calbiochem (La Jolla, CA). The following antibodies were used: anti-Akt, anti-phospho-Akt (Ser473); New England Biolabs, Beverly, MA; anti-phospho-MAPK (Promega, Madison, WI); anti-caveolin-1 and anti-CD31 (Transduction Laboratories, Lexington, KY); and anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-AMF and anti-AMFR were described previously (17, 31). Anti-AMF IgG and preimmune IgG were purified from serum using Immunopure (G)IgG according to the manufacturer’s instructions (Pierce, Rockford, IL).

Cell Culture and Transfection. NIH-3T3 cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C and 5% CO2. The full-length human AMF/PGI cDNA was generated by PCR amplification (17). The PCR product was ligated into a mammalian expression vector pcDNA3.1 zenovo (+; Invitrogen, Carlsbad, CA). Parental NIH-3T3 cells were transfected with AMF/PGI cDNA using LipofectAMINE according to the manufacturer’s instructions (Life Technologies, Inc., Gaithersburg, MD). Isolation of single clones of the stable transfectants were accomplished by adding Zeocin (Invitrogen) to the culture medium at 750 μg/ml.

Northern Blot Analysis. Total cellular RNA was isolated using Trizol reagent (Invitrogen), and 20 μg of RNA were analyzed via Northern hybridization using 32P-labeled AMF/PGI cDNA probe (17).

Western Blot Analysis. Cell lysates were separated by SDS-PAGE and analyzed by Western blotting as described previously (17).

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day. To confirm the role of the AMF/PGI in the tumor cell growth in soft agar, cells were seeded in soft agar containing the anti-AMF IgG or preimmune IgG. Colonies developed in the agar suspension were examined 3 weeks after seeding, and the number of colonies counted and photographed using phase-contrast photomicrography. Colonies measuring ≥0.1 mm in diameter were scored. Results were expressed as the percentage of colonies formed per total number of seeded cells. Each experiment was performed in triplicate.

Female athymic nude mice were housed under specific pathogen-free conditions and used at 6 weeks of age. To investigate tumorigenicity in nude mice, each mouse was inoculated on both flanks with 1 x 10^5 cells in 0.1 ml of PBS per site. After xenografts became visible, the sizes of xenografts were determined every 3 days by externally measuring tumors in two dimensions. The volume (V) of the xenograft was calculated by the following equation: V = (L x W^2) x 0.5, where L is the length and W is the width. The mean values and the SDs of the tumor volumes were calculated. Five mice were inoculated at both sides of the flank with each type of cells in two separate experiments.

**Histopathological and Immunohistochemical Study.** The tumors were fixed in 10% phosphate-buffered formalin, and paraffin-embedded 4 μm-thick sections were prepared. Slides were stained with H&E according to standard laboratory protocols. Immunohistochemical study was performed using Vectastain Elite ABC kit according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA).

**Invasion Assay.** The invasive activity of cells was assayed in Transwell cell culture chambers (Corning Costar Co., Cambridge, MA). Polycarbonate filters with 8.0-μm pore size were coated with Matrigel (Collaborative Bio- medical Products, Bedford, MA; 1 mg/ml) to form a matrix barrier. Cells were resuspended to a concentration of 1 x 10^5/ml cells in DMEM with 0.5% FBS. The cell suspension (100 μl) was added to the upper compartment of the chamber, and incubated with DMEM with 10% FBS in the lower compartment for 24 h at 37°C. The filters were fixed with 4% paraformaldehyde and stained with Hema 3 (Fisher Scientific, Pittsburgh, PA). The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells that had invaded through Matrigel and the filter to the lower surface were counted. Each assay was performed in triplicate.

**Cell Proliferation Assays.** Cell proliferation assays were performed by seeding the AMF/PGI or empty vector-transfected cells at a density of 1 x 10^5 cells/well in six-well plates. Cells were fed DMEM with 10% FBS every other day, and the number of cells in the wells were manually counted with a hemocytometer. For the serum dependence assay, cells were seeded at a density 1 x 10^5 cells/well in DMEM with 10% FBS. The next day, the medium was changed to serum-free DMEM and maintained in DMEM without FBS. In some experiments, anti-AMF IgG or preimmune IgG was incubated in the medium.

**Induction of Apoptosis by MAPK or and PI3K Inhibition.** Cells (1 x 10^5) were seeded in six-well plates. The next day, medium was replaced with serum-free medium containing inhibitors or vehicle controls. PD98059 and LY294002 were reconstituted in DMSO. Cells were treated with PD98059 (50 μM) for 24 h in serum-free condition. Treatment with LY294002 (50 μM) was carried out for 4 h, the medium was changed to serum-free medium without inhibitor, and cells were incubated for 24 h in serum-free conditions. Cells were harvested and the percentage of cell survival determined by trypan blue dye exclusion. Subsequently, DNAs were isolated, separated in 1.8% agarose gel, and visualized by ethidium bromide staining.

**Statistical Analysis.** Statistical analyses were determined using a Student’s t test. P < 0.05 was considered significant.

**RESULTS**

**Constitutive Overexpression of AMF/PGI in NIH-3T3 Transfectants.** An expression vector containing the Zveicin-resistance gene and the AMF/PGI cDNA was transfected into the NIH-3T3 cells. Individual Zeocin-resistant cell clones were isolated, and stable AMF/PGI protein-expressing clones were verified by Western blot analysis. Three clones exhibiting high level expression of AMF/PGI were selected. The ratios of AMF/PGI expression was detected by densitometry and were 6.6 (clone 1), 6.5 (clone 2), and 5.3 (clone 3) compared with the empty vector-transfected control (Fig. 1A). The expression levels of AMF/PGI mRNA were determined by Northern blot analysis using full-length AMF/PGI cDNA as a probe. The three selected clones expressed high levels of AMF/PGI mRNA, whereas empty vector-transfected cells expressed AMF/PGI at the same level as that of untransfected NIH-3T3 cells. Measurement of the expression of AMF/PGI mRNA revealed ratios of 2.3 (clone 1), 2.6 (clone 2), and 2.1 (clone 3), relative to empty vector-transfected cells (Fig. 1B). To investigate the secretion levels of AMF/PGI, subconfluent cells were incubated with serum-free DMEM for 24 h and 50 μg of protein from conditioned medium analyzed by Western blotting (17). AMF/PGI secretion was restricted to the transfectants, and could not be detected in the conditioned medium of the parental and vector-only transfected NIH-3T3 cells (Fig. 1A). Next, we questioned whether the expression of the ligand was associated with the elevated level of its receptor, and aliquots of cell lysates were subjected to quantitative Western blot analysis using anti-AMFR antibody. No differences in expression of AMFR were observed between AMF/PGI-transfectants and vector-only transfected cells (Fig. 1A). The experiment was performed in triplicate using different protein preparations.

**AMF/PGI Overexpression Is Transforming and Induces Tumorigenesis.** AMF/PGI transfection induced cellular morphological changes including a fusiform appearance and colony formation typical of cell transformation (Fig. 2, c and c'). Almost complete reversion into the flat phenotype (Fig. 2, d and d') was seen in AMF/PGI-transfected NIH-3T3 cells after administration of the anti-AMF IgG, but not by preimmune serum (data not shown), suggesting that the morphological change was caused by the overexpression of AMF/PGI. The morphology of the parental NIH-3T3 cells (Fig. 2, a and a') was similar to that of the vector-only transfected cells (Fig. 2, b and c).
The morphology of parental NIH-3T3 cells treated with anti-AMF IgG (Fig. 2, e and e') was almost the same as that of NIH-3T3 cells without anti-AMF IgG (Fig. 2, a and a'). In contrast with parental and vector-only transfected NIH-3T3 cells, AMF/PGI-transfected NIH-3T3 cells acquired the ability of anchorage-independent growth in soft agar (not shown). Furthermore, anti-AMF IgG antibody but not pre-immune IgG suppressed the colony formation of AMF/PGI-transfected cells when included in the soft agar medium confirming a role for secreted protein in the enhancement of anchorage-independent growth of cells by AMF/PGI overexpression.

Secretion of AMF/PGI was selectively detected in the conditioned medium of ras-transformed but not untransformed NIH-3T3 cells (12), and a ras and abl transformation of NIH-3T3 cells was shown to be associated with significant reduced expression of caveolin (29, 32). As seen in Fig. 3, Western blot analysis of the expression of caveolin in parental, vector-only transfected, and three independent AMF/PGI-transfected NIH-3T3 cell clones revealed that the level of caveolin and caveolin-1 in AMF/PGI-transfected cell clones was reduced by approximately 30–40% relative to parental NIH-3T3 cells confirming the above reports, and supporting the function of AMF/PGI is a transforming protein. To deduce other aspects of transformation affected by AMF/PGI overexpression, we tested the invasive capacity by the cells in Matrigel using Transwell culture plates assay. The invasive potential was 2–2.5 times greater in AMF/PGI-transfected cells compared with parental or vector-only transfected NIH-3T3 cells at 24 h (Fig. 4A). Invasive capacity was specifically inhibited by anti-AMF IgG (Fig. 4B). The ability of anti-AMF antibody to revert the morphological changes, and the enhanced growth, invasiv-

Fig. 2. Morphological changes in growth of AMF/PGI-transfected NIH-3T3 cells. Single clones of transfected NIH-3T3 cells were selected by Zeocin. The morphology of the parental NIH-3T3 cells (a and a') did not differ significantly from that of the empty vector-transfected cells (b and b'). However, morphological changes (fusiform appearance) and colony formation typical of the transforming growth were observed in the AMF/PGI transformants (c and c': clone 1). Morphological changes of AMF/PGI-transfected cells were inhibited by anti-AMF IgG (50 μg/ml; d and d'). Parental NIH-3T3 cells treated with anti-AMF IgG are shown. e and e', All cells are shown at ×40 (left column) and ×100 (right column) magnification.

Fig. 3. Caveolin-1 expression in AMF/PGI-transfected NIH-3T3 cells. Cell lysates from parental, vector-only transfected, and three independent AMF/PGI-transfected NIH-3T3 cells were subjected to Western blot analysis with antibodies to caveolin and caveolin-1. The amount of caveolin and caveolin-1 protein in AMF/PGI-transfected cells reduced 60–70% of levels found in parental NIH-3T3 cells by densitometric tracing analysis; bars, ±SD.

Fig. 4. Invasive ability of AMF/PGI-transfected cells. A, Two-hundred μl of a single-cell suspension (1 × 10⁶ cells/ml) of cells were placed in the upper wells of individual Transwell inserts containing 8-μm pore polycarbonate membranes precoated with Matrigel. Cells were allowed to invade for 24 h at 37°C, and then they were fixed and stained with Hema-3. Cells on the upper surface were removed with a cotton swab, and the cells that migrated to the lower side of the membrane were mounted onto a microscope slide and counted under a light microscope at ×200 magnification. In vitro invasive ability was significantly greater in AMF/PGI-transfected cells when compared with parental and vector-only transfected NIH-3T3 cells at 24 h. B, invasive capacity was inhibited by anti-AMF IgG (50 μg/ml). Data shown are the mean values from three triplicate experiments for each group; bars, ±SE, *, significant difference when compared with control (P < 0.05).
Fig. 5. A, transfected cells and parental NIH-3T3 cells (1 × 10^6) were s.c. inoculated into the back of 6-week-old female athymic nude mice. Five mice were inoculated at both sides with each type of cells in two separate experiments. Tumor formation was monitored for up to 4 weeks. The two independent experiments produced similar results. B, pathological characteristic of AMF/PGI-induced tumors in nude mice. Tumors were generated by inoculation of AMF/PGI-transfected NIH-3T3 cells as described in “Materials and Methods.” Histological appearance of xenografts tumors from mice that were inoculated with AMF/PGI-transfected cells. Tumor consisted of spindle-shaped cells and included fusiform tumor cells that exhibited markedly pleomorphic hyperchromatic nuclei and mitosis (Fig. 5B, panel a), and included fusiform tumor cells that exhibited markedly pleomorphic hyperchromatic nuclei and mitosis (Fig. 5B, panel b). Histological examination identified the tumors as fibrosarcomas. Immunohistochemical staining for antimouse CD31 were performed to establish tumor angiogenesis and, indeed, many microvessels were detected in the growing tumors (Fig. 5B, panel c).

AMF/PGI Overexpression Confers Protection against Apoptosis via the PI3K/Akt Pathway. Each of the AMF/PGI-transfected cell clones grew at rates faster than those of parental and vector-only transfected NIH-3T3 cells (Fig. 6A). The increase in cell number of the AMF/PGI-transfected cell clones was 2-fold greater than the control cells after 7 days in culture. Notably, whereas serum deprivation results in rapid cell death of parental and vector-only transfected NIH-3T3 cells (95% of these cells die within 72 h), approximately 80–90% of AMF/PGI-transfected cells survive after serum withdrawal and continue proliferating in serum-free conditions (Fig. 6B). The effect of anti-AMF IgG on cell proliferation of parental and AMF/PGI-transfected NIH-3T3 cells was determined by growth curve analysis as demonstrated in Fig. 6C. Anti-AMF IgG (50 μg/ml) inhibited parental, vector-only transfected, and AMF/PGI-transfected NIH3T3 cell proliferation by 19%, 21% and 33%, respectively, after 7 days of cultivation compared with control. Growth inhibition of anti-AMF IgG on AMF/PGI-transfected NIH-3T3 cells was significantly greater than that on parental and vector-only transfected NIH-3T3 cells (Fig. 6, A and C). Preimmune rabbit IgG had no effect on proliferation in cultures of these cell lines (data not shown).

Pharmacological inhibitors were used to determine the signaling pathway(s) activated by AMF/PGI overexpression that inhibit apoptosis. Survival of the AMF/PGI-transfected cells under serum deprivation was specifically inhibited by the PI3K inhibitor LY294002 and relatively resistant to growth inhibition by PD98059 inhibition of MAPK kinase activation (Fig. 7A). Inhibition of both pathways simultaneously showed only a limited additive effect on serum starvation-induced apoptosis. Induction of oligonucleosomal DNA fragmentation, an indicator of apoptosis, occurred in parental and vector-only transfected NIH-3T3 cells after 24 h of serum withdrawal alone and when treated with PD98059 and/or LY294002 (data not shown). In contrast, DNA fragmentation could be detected in AMF/PGI-transfected NIH-3T3 cells when treated with LY294002 but not with PD98059 (Fig. 7B). Therefore, AMF/PGI-induced apoptosis protection is mainly mediated by the PI3K/PKB pathway.

PKB/Akt is catalytically inactive in serum-starved primary and immortalized fibroblasts (35). The activation of PKB/Akt results from phosphorylation of both Thr308 and Ser473 (34). Using phosphospecific antibodies revealed that on serum deprivation the PBK/Akt phosphorylation levels were increased only in AMF/PGI-transfected cells and not in vector-only transfected or parental NIH-3T3 cells (Fig. 8A). The phosphorylation of PBK/Akt was abrogated by inhibition of PI3K with pharmacological inhibitor LY294002 demonstrating the involvement of PI3K-mediated signaling in AMF/PGI-induced PKB/Akt activation. The MAPK pathway has also been identified as an important signaling pathway activated by various growth factors (35). Using the phospho-MAPK antibody, MAPK activation was equivalent in untransfected and AMF/PGI-transfected NIH-3T3 cells (Fig. 8B). The ability of the MAPK kinase inhibitor PD98059 to inhibit phosphorylation of MAPKs indicates that the MAPK pathway
probably does not regulate AMF/PGI-mediated cell survival. In conclusion, AMF/PGI overexpression and secretion induce NIH-3T3 cell transformation, invasion, and tumorigenicity associated with apoptosis resistances via PI3K-dependent activation of PKB/Akt phosphorylation.

**DISCUSSION**

**AMF/PGI Overexpression Is Transforming.** The transforming ability of the glycolytic enzyme AMF/PGI to induce cell transformation and tumorigenesis represents the first demonstration of the oncogenic potential of overexpression and secretion of this housekeeping protein. Expression of AMF/PGI and other glycolytic enzymes are up-regulated in response to enhanced energy requirements under hypoxic condition (36). However, AMF/PGI is different from other glycolytic enzymes in that it acts as a moonlighting protein that also exhibits cytokine activity when secreted from cells. AMF/PGI gene expression has been shown to be developmentally regulated in brain (37) as well as induced during differentiation of osteoblast cells (38), and to be elevated under hypoxic conditions (39, 40). The demonstration that AMF/PGI-overexpressing NIH-3T3 cells exhibit enhanced growth properties in vitro, anchorage-independent growth, and tumorigenicity in vivo highlights the possible role of AMF/PGI overexpression during normal cellular responses, and its contribution to cellular transformation.

Anti-AMF antibodies abrogate the altered morphology, enhanced growth, increased invasivity, and acquisition of anchorage-independent growth of the AMF/PGI-overexpressing NIH-3T3 cells; thus, the acquired phenotype is a direct consequence of its secretion and autocrine activation of the AMFR, and probably not because of enhanced glycolytic function. The cytokine function of the enzyme was acquired over evolution because only mammalian and not bacterial or yeast PGIs express cytokine activity (41) and may represent a prosurvival mechanism to protect glycolytically active cells from premature death. Similarly, glycolysis is generally up-regulated in tumor cells, and is an essential element of tumor cell motility and invasion.

**Fig. 6. Growth of AMF/PGI-transfected NIH-3T3 cells.** Cells (1 x 10^3/well) were seeded into six-well plates in DMEM with 10% FBS. The next day, the medium was replaced by DMEM with or without 10% FBS. Cells were fed with fresh medium every other day, and the number of cells in the wells were manually counted with a hemocytometer daily for up to 7 days after seeding. A, AMF/PGI-transfected cells grew faster than the parental and empty vector-transfected cells in DMEM with 10% FBS. B, AMF/PGI-transfected cells survived in serum-free conditions. Serum-deprivation resulted in rapid cell death of control NIH-3T3 cells and empty vector-transfected cells. AMF/PGI-transfected cells were resistant to serum withdrawal. Approximately 80–90% of cells survived, and continued growing in serum-free conditions. C, influence of antibody to the AMF/PGI on the growth of cells in DMEM containing 10% FBS. Anti-AMF IgG (50 μg/ml) inhibited parental, vector-only transfected, and AMF/PGI-transfected NIH-3T3 cell proliferation by 19%, 21%, and 33%, respectively, after 7 days of cultivation compared with control. Growth inhibition effect of anti-AMF IgG on AMF/PGI-transfected NIH-3T3 cells was significantly greater than that on parental and vector-only transfected NIH-3T3 cells. Preimmune rabbit IgG had no effect on proliferation in cultures of these cell lines (data not shown). Each bar represents the mean of triplicate wells ± SD. The experiment was repeated twice with similar results. (●), parental NIH3T3; (∆), empty vector-transfected NIH3T3; (○), AMF/PGI-transfected clone 1; (△), clone 2; (□), clone 3.

**Fig. 7. Signal pathways involved in protection against apoptosis induced by serum-deprivation.** A, figure shows that on serum starvation the AMF/PGI-transfected cell-induced cell survival is decreased by LY294002, but the AMF/PGI-transfected cells were relatively resistant to growth inhibition by PD98059. Simultaneous treatment with both inhibitors showed an additive effect. No differences were observed in vector-only transfected NIH-3T3 cells that were treated with exogenous AMF/PGI. (○), serum deprivation; (●), serum deprivation + PD98059; (◆), serum deprivation + LY294002; (□), serum deprivation + PD98059 + LY294002. *, significant difference when compared with control (P < 0.05); bars, ± SD. B, induction of oligonucleosomal DNA fragmentation treated with PD98059 and LY294002 in AMF/PGI-transfected NIH-3T3 cells. Cells were cultured in serum-free condition. Treatment with PD98059 (50 μM) was carried out for 24 h and for 4 h with LY294002 (50 μM). DNA was isolated, subjected to agarose gel electrophoresis, and visualized by ethidium bromide staining. DNA fragmentation was only observed under LY294002 treatment. These results indicate that AMF/PGI overexpression-induced protection may be mainly mediated by the PI3K/PKB pathway. Lane 1, standard marker (1 kb ladder). Lanes 2, 5, and 8, AMF/PGI-transfected clone 1. Lanes 3, 6, and 9, AMF/PGI-transfected clone 2. Lanes 4, 7, and 10, AMF/PGI-transfected clone 3.
expression is directly regulated by hyperactivation of p42/44\(\text{MAPK}\) cascade (51). Here, we show that p42/44\(\text{MAPK}\) is not activated in response to autocrine AMF/PGI stimulation suggesting that overexpression of AMF/PGI may stimulate reduction in caveolin-1 levels via a different pathway. AMFR is internalized to the endoplasmic reticulum via a caveolin-dependent pathway that is negatively regulated by caveolin-1 expression (32, 52). The autocrine activation loop by AMF/PGI of its receptor may therefore occur in the caveolae, and its stimulation acts to regulate caveolae internalization and stabilization at the plasma membrane, and potentially affect caveolin expression levels.

**Secreter AMF/PGI Is Antiapoptotic.** It was shown recently that AMF/PGI acts as an angiogenic factor in vivo and in vitro (46). AMF/PGI stimulates in vitro motility of human umbilical vein endothelial cells, elicited the formation of tube-like structures mimicking angiogenesis when human umbilical vein endothelial cells were grown in three-dimensional type I-collagen gels, and specific PGI inhibitors prevented angiogenic activity and neovascularization in the mouse model. Here, staining of the developing with endothelial cell marker (antimouse CD31) identified many microvessels in AMF/PGI-transfected tumors supporting the notion that a tumor secreted AMF/PGI may act as an angiogenic factor that acts in a paracrine manner.

Human basic FGF (FGF-2) is a prototype member of the large family of heparin-binding growth factor genes that regulates various biological processes, such as proliferation, differentiation, migration, angiogenesis, and survival in different cells (25). Similar to PGI, FGF-2 is expressed in a broad range of tumor cells; its expression and its release is modified during tumor progression (53). FGF-2 also induces endothelial cell proliferation, migration, and angiogenesis in vitro, and regulates the expression of several molecules that mediate angiogenesis (25). Overexpression of FGF-2 in NIH-3T3 fibroblasts modulates Bcl-2 (54) and interleukin 6 levels (55) leading to inhibition of apoptosis through alternate molecular mechanisms. The \(M_r\) 18,000 low molecular weight isoform (LMW FGF-2) is cytosolic and like AMF/PGI is also secreted via an endoplasmic reticulum-Golgi-independent pathway; therefore, this isoform acts as a paracrine/autoocrine factor. In contrast, the high molecular weight isoforms of FGF-2 (HMW FGF-2) contain nuclear localization sequence-like signals responsible for nuclear targeting (25). The selective expression of either LMW or HMW FGF-2 forms induces NIH-3T3 cell transformation as measured by enhanced saturation density and growth in soft agar (56). Therefore, AMF/PGI and FGF are both angiogenic factors secreted via the nonclassical pathway that exhibit similar biological functions. Like FGF-2, PGI transfectants possess an enhanced proliferative potential under serum-free conditions and antiapoptotic ability. The data indicate that AMF/PGI protects cells from apoptosis, and that the specific PI3K inhibitor LY294002 sensitizes cells to apoptosis and abolishes protection. Inhibition of apoptosis by AMF/PGI is associated with kinase activity of the antiapoptotic mediator Akt. The PI3K/Akt pathway is activated in response to many stimuli resulting in an antiapoptotic effect (57). On the basis of this, Akt was determined to be an antiapoptotic regulating protein through which various survival signals suppress cell death (58). The activation of the PI3K/Akt pathway is required for cell spreading and matrix-induced cell survival, and integrin-mediated adhesion activates Akt in a PI3K-dependent manner (59).

MAPK was shown to be involved in the protection of cells from apoptosis induced by growth factor withdrawal (60). IGF-I is capable of preventing apoptosis by activation of multiple signal transduction pathways including PI3K and MAPK pathways. These two pathways appear to interact with each other and to a certain extent may replace each other (22). FGF-2 stimulated DNA synthesis and induced a sustained phosphorylation of p42/44\(\text{MAPK}\) in rat mammary fibroblasts.
In conclusion, overexpression and consequent secretion of AMF/PGI constitutively activates its receptor via an autocrine loop that induces cellular transformation, tumorigenicity, and apoptosis survival through the activation of the PI3K/Akt signaling pathway. The protection of cells from apoptosis by this ubiquitous glycolytic enzyme suggests that the alternate cytokine function of this moonlighting protein may have been acquired to protect active glycolytic cells from cell death under hypoxic condition of growing tumors.

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