pH-specific sequestration of phosphoglucose isomerase/autocrine motility factor by fibronectin and heparan sulphate

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Summary

Phosphoglucose isomerase (PGI) is a glycolytic enzyme that moonlights as a cytokine under the aliases autocrine motility factor (AMF), neuroleukin and maturation factor. The cytokine function of PGI/AMF targets multiple cell types however mechanisms that regulate and sequester this ubiquitous, circulating cytokine remain largely unidentified. PGI/AMF is shown here to exhibit fibronectin (FN)-dependent cell surface association at both neutral and acid pH. Direct PGI/AMF binding to FN and fluorescence resonance energy transfer (FRET) between PGI/AMF and FN were detected only at pH 5. At neutral pH, the interaction of PGI/AMF with FN is receptor-mediated requiring prior clathrin-dependent endocytosis. PGI/AMF and FN do not co-internalize and PGI/AMF undergoes a second round of endocytosis upon recycling to the plasma membrane indicating that recycling PGI/AMF receptor complexes associate with FN fibrils. Heparan sulphate does not affect cell association of PGI/AMF at neutral pH but enhances the FN-independent cell surface association of PGI/AMF at acid pH identifying two distinct mechanisms for PGI/AMF sequestration under acidic conditions. However, only PGI/AMF sequestration by FN at acid pH was able to stimulate cell motility upon pH neutralization identifying FN as a pH-dependent cytokine trap for PGI/AMF. The multiple ways of cellular association of PGI/AMF may represent acquired mechanisms to regulate and harness the cytokine function of PGI/AMF.

Key words: Cytokine sequestration, Cell motility, Extracellular matrix

Introduction

Phosphoglucose isomerase (PGI, EC 5.3.1.9) is a glycolytic enzyme expressed in all cells that plays an essential role in gluconeogenesis. It represents an example of a moonlighting protein that also functions as an extracellular cytokine (Copley, 2003; Jeffery, 1999; Smalheiser, 1996). The neurokine, neuroleukin, was identified in 1986 and shown to increase the survival of cultured sensory neurons and, following secretion by lectin-stimulated T cells, to induce the maturation of B-cells into antibody secreting cells (Gurney et al., 1986a; Gurney et al., 1986b). Subsequent comparison of the sequence of neuroleukin with that of PGI revealed identity between the two proteins, raising the question as to how a ubiquitous, cytosolic enzyme could also function as an extracellular cytokine (Chaput et al., 1988; Faik et al., 1988). The demonstration in 1996 that autocrine motility factor (AMF), a tumor secreted cytokine, and maturation factor, a lymphokine, were identical to PGI confirmed the extracellular moonlighting cytokine function of this glycolytic enzyme (Watanabe et al., 1996; Xu et al., 1996). The association of hippocampal expression of PGI/AMF and its receptor [gp78 (glycoprotein of 78 kDa) or AMFR (autocrine motility factor receptor)] with learning and memory (Luo et al., 2002) and the ability of PGI/AMF to induce the differentiation of human myeloid cells into monocytes (Xu et al., 1996) further supported the previously reported neurokine and lymphokine functions of PGI/AMF (Gurney et al., 1986a; Gurney et al., 1986b). PGI/AMF cytokine activity has also been shown to be involved in osteoblast differentiation during mineralization (Zhi et al., 2001) and to be necessary for embryo implantation (Schulz and Bahr, 2003). PGI/AMF is therefore a ubiquitous cytokine with multiple cellular and tissular targets.

As AMF, PGI was identified as a cytokine secreted by tumor cells whose expression is linked to tumor cell invasion and metastasis (Liotta et al., 1986; Watanabe et al., 1996). Serum PGI/AMF activity has long been reported and is associated with tumor expression (Bodansky, 1954; Schwartz, 1973) indicating that, although the mechanism remains obscure, this protein is actively released from both normal and tumor cells. Overexpression of PGI/AMF by stable transfection of NIH-3T3 cells has shown that its de novo secretion is sufficient for cellular transformation and tumorigenicity (Tsutsumi et al., 2003). PGI/AMF also stimulates angiogenesis via the paracrine stimulation of vascular endothelial growth factor receptor (VEGFR) Flt-1 expression (Funasaka et al., 2001; Funasaka et al., 2002). Interestingly, both PGI/AMF and VEGF have

recently been reported to exhibit pH dependent binding to fibronectin (FN) (Amraei et al., 2003; Goerges and Nugent, 2003; Goerges and Nugent, 2004). pH-dependent association with FN may serve to sequester angiogenic cytokines under conditions of hypoxia and consequent acidification that can subsequently serve to induce angiogenesis towards the damaged region (Goerges and Nugent, 2004).

PGI/AMF cytokine activity is found in rheumatoid synovial fluid (Watanabe et al., 1994) and its deposition on synovial surfaces and ability to induce an autoimmune response in rheumatoid arthritis (RA) identified it as a possible autoantigen different from normal circulating PGI/AMF (Mandik-Nayak et al., 2002; Matsumoto et al., 1999; Schaller et al., 2001). Our demonstration that acid-induced changes in the tertiary structure of PGI/AMF strongly increase its association with the extracellular FN matrix argues that changes in local microenvironment could alter the behavior of the protein (Amraei et al., 2003). Indeed, intra-articular hypoxia in rheumatoid joints and consequent upregulation of PGI/AMF has been suggested to perpetuate RA (Naughton, 2003). Involvement of PGI/AMF in RA may represent a pathological exaggeration of a normal biological process, the acid-mediated sequestration of cytokines by the extracellular matrix (ECM). However, in spite of the well-characterized ability of ECM-associated proteoglycans, such as heparin, to sequester cytokines (Ruoslahti et al., 1992; Vlodavsky et al., 1996) alternate mechanisms by which the ECM sequesters cytokines remain poorly defined.

The PGI/AMF receptor, gp78 or AMFR, is a seven-transmembrane domain G protein-coupled receptor recently identified as an endoplasmic reticulum (ER)-associated RING finger-protein ligase (Fang et al., 2001; Registre et al., 2004; Shimizu et al., 1999). AMFR internalizes its ligand via both caveolae/raft-dependent endocytosis to the smooth ER and clathrin-dependent endocytosis to multivesicular bodies (Benlimame et al., 1998; Le et al., 2000; Le et al., 2002; Le and Nabi, 2003; Nabi and Le, 2003). The clathrin-dependent pathway has been shown to be associated with the recycling of PGI/AMF and its receptor to cell surface FN fibrils (Le et al., 2000). The ability of PGI/AMF to associate with FN upon conformational changes at acid pH (Amraei et al., 2003) as well as following receptor-mediated recycling at neutral pH (Le et al., 2000) led us to address the specificity and mechanisms of PGI/AMF association with FN fibrils at neutral and acid pH. At neutral pH, the interaction of PGI/AMF with FN fibrils is receptor-mediated and requires prior endocytosis and recycling. At acid pH, PGI/AMF binds directly to FN and also exhibits a dose-dependent FN-independent increase in cell surface binding in the presence of heparan sulphate (HS) identifying two distinct mechanisms for PGI/AMF sequestration at acid pH. Acid-dependent interaction of PGI/AMF with FN but not HS was able to stimulate cell motility upon pH neutralization suggesting that under conditions of low pH, such as tumor hypoxia, direct interaction with FN sequesters this angiogenic factor.

Materials and Methods

Antibodies and reagents

Rabbit phosphoglucose isomerase (Sigma P-9544; referred to as PGI/AMF), poly-L-lysine, bovine plasmatic FN, bovine serum albumin solution (BSA 30%), and heparan sulphate proteoglycan were purchased from Sigma Chemical Co. (Oakville, ON). Monoclonal anti-FN antibody was purchased from Transduction Laboratories (Mississauga, ON). Streptavidin conjugated to either Texas Red or fluorescein was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse anti-hemagglutinin (HA) was a gift from Luc Desgroseillers (Department of Biochemistry, Université de Montréal). Monoclonal Anti-T7 tag antibody was purchased from Novagen. Texas-Red conjugated phalloidin, Alexa-488 goat anti-mouse, Alexa-568 goat anti-mouse, and Alexa-647 goat anti-rabbit secondary antibodies were purchased from Molecular Probes (Eugene, OR). Human FN-fluorescein isothiocyanate (FITC) was a gift from Deane Mosher (University of Wisconsin, Madison, WI). The Alexa Fluor 488, Alexa Fluor 568 and the FluoReporter® FITC Protein Labeling Kits were purchased from Molecular Probes (Eugene, Oregon) and used to tag both PGI/AMF and bovine FN according to the manufacturer's instructions. PGI/AMF was biotinylated with long chain N-hydroxyl-succinimido-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions.

Cell culture

NIH-3T3 fibroblasts obtained from the American Type Culture Collection (1658-CRL) were cloned for these studies (Benlimame et al., 1998). Fibroblasts differentiated from ES cells heterozygous ($FN^{+/-}$) and homozygous ($FN^{-/-}$) for the FN mutation (Saoncella et al., 1999) were obtained from Deane Mosher. The cells were grown in a Dulbecco's modified Eagle's medium (DMEM) supplemented with either 10% calf serum (NIH-3T3) or 10% fetal bovine serum (FBS) ($FN^{+/-}$ and $FN^{-/-}$ fibroblasts), non-essential amino acids, vitamins, glutamine and a penicillin-streptomycin antibiotics (Canadian Life Technologies) at 37°C in a humidified 5% CO_2 95% air incubator. Where indicated, cover slips were coated with FN by incubation with 200 μl of a solution of 10 μg ml^{-1} of FN in PBS for 1 hour at room temperature and then washed three times with PBS before plating the cells.

Viral infection

Recombinant adenovirus expressing the tetracycline-regulated chimeric transcription activator (tTA), HA-tagged wild-type dynamin-1 (dynWT), HA-tagged dominant negative dynamin-1 K44A mutant (dynK44A) and T7-tagged clathrin hub (Cla-hub), under the control of the tetracycline-regulated promoter were used as previously described (Altschuler et al., 1998; Altschuler et al., 1999; Le et al., 2002). To enhance infection rates, viral stocks of tTA, dynWT, dynK44A and Cla-hub, were pre-incubated with 10 μg ml^{-1} of poly-L-lysine for 30 minutes at room temperature. Infection with only tTA adenovirus was used as a control. NIH-3T3 cells were plated on glass cover slips for 24 hours and rinsed once with PBS before addition of the adenovirus/poly-L-lysine mixture in 2 ml of serum-free medium for 1 hour at 37°C. After removal of the adenovirus mixture, the cells were rinsed twice with serum-free media and then incubated for 36 hours in regular culture media.

Immunofluorescence labeling

Fifty-thousand NIH-3T3, $FN^{+/-}$ and $FN^{-/-}$ fibroblasts were plated on glass cover slips for 2 days before each experiment. For the PGI/AMF internalization studies, cells were pulse labeled with 5 or 25 μg ml^{-1} of biotinylated PGI/AMF (bPGI/AMF) or Alexa-568 conjugated PGI/AMF (PGI/AMF-568) in bicarbonate-free, 100 mM HEPES-supplemented (pH 7.4) complete medium (complete HEPES medium) or in bicarbonate-free, 100 mM MES-supplemented (pH 5.0) complete medium for the indicated times at 37°C. Where indicated cells were pretreated with 10 μg ml^{-1} heparan sulphate proteoglycan

for 1 hour, competed with excess (100 μg ml^{-1}) PGI/AMF, or co-incubated with 10 μg ml^{-1} fluorescein conjugated fibronectin (FN-FITC). Cells were washed with complete HEPES medium and PBS (pH 7.4) supplemented with 0.1 mM Ca^{2+} and 1 mM Mg^{2+} (PBS-CM) and then fixed with 3% paraformaldehyde for 15 minutes at room temperature. After fixation, cells were rinsed extensively with PBS and permeabilized with 0.1% Triton X-100 in PBS-CM containing 0.5% BSA (PBS-CM/TX/BSA) for 15 minutes to reduce non-specific binding. All washings and incubations with both primary and secondary antibodies were done with PBS/CM containing 0.2% BSA (PBS-CM/BSA). Postfixation labeling was performed with Texas Red-streptavidin to label bPGI/AMF or with the indicated primary antibodies followed by the appropriate fluorescent secondary antibodies. To follow the recycling of PGI/AMF, NIH-3T3 cells were pulse labeled with 25 μg ml^{-1} of bPGI/AMF for 30 minutes at 37°C in complete HEPES medium, washed with complete medium during 10 minutes and then incubated with Texas Red-streptavidin at 37°C or at 4°C for 30 minutes. The cells were washed extensively with complete HEPES medium and PBS-CM before fixation with 3% paraformaldehyde and permeabilization with PBS-CM/TX/BSA. Intracellular bPGI/AMF was then labeled with FITC-streptavidin for 30 min. After labeling, the cover slips were mounted in Airvol (Air Products Inc, Allentown, PA) and viewed with the 60× Plan Apochromat objective of a BioRad MRC-600 or the 63× Plan Apochromat objective of a Leica TCS-SP1 confocal microscope equipped with 488, 568 and 633 laser lines.

From 8-bit confocal images, acquired with the 63× objective of the Leica TCS SP1 confocal microscope at zoom 1, of cells double labeled for PGI/AMF-568 and FN, cell- and FN-fibril associated fluorescence intensity were quantified using Northern Eclipse software (Empix Imaging, Mississauga, Ontario). For each experiment, using the most intensely labeled PGI/AMF-568 slide, gain and offset were adjusted (glowoverunder) to just below saturation and just above zero intensity, respectively, and all images were subsequently acquired with the same confocal settings. From 10 images per condition, non-cellular regions were cut and total fluorescence intensity of the PGI/AMF-568 label determined. Subsequently, a Boolean AND operation was performed between the PGI/AMF-568 labeled image and the FN image and the fluorescent intensity of only the PGI/AMF-568 positive pixels that overlapped with FN labeled pixels quantified from each image to provide a measure of the PGI/AMF labeling that overlapped with FN fibrils. Presented data represent the average of at least three independent experiments (±s.e.m.) and statistical analyses were performed using the Student's *t*-test.

Photobleaching fluorescence resonance energy transfer (FRET)

Photobleaching FRET analysis was performed on fixed cells by measuring donor fluorescence intensity before and after destruction of the acceptor by photobleaching. NIH-3T3 cells were incubated with FN-488 for 30 minutes at pH 7.5 in complete medium and then for 30 minutes with PGI/AMF-568 at pH 7.5 or at pH 5 in bicarbonate-free medium at 37°C. Incubations with only FN-488 or PGI/AMF-568 or with FN-488 and FN-568 at pH 7.5 served as controls. Cell were washed with PBS/CM and fixed with 3% paraformaldehyde and the cover slips were mounted in Airvol. Images were collected and analysed on a Leica TCS-SP1 confocal microscope (Leica Microsystems, Germany) equipped with a 63× Plan Apochromat objective, an argon laser for 488 nm excitation, a krypton laser for 568 nm excitation and data analysis software to quantify the intensity of the fluorescence.

The measured FRET efficiency was calculated using the technique of acceptor photo-bleaching. First, single scan images at zoom 1× of donor (Alexa-488) and acceptor (Alexa-568) were collected with a pinhole of 1 Airy unit. Then, the acceptor was bleached in the zone of interest with the Krypton 568 laser at high intensity for 20 frames over 32 seconds 395 milliseconds such that the acceptor was photo-bleached by 60% to 90%. New single scan images at zoom 1× were then acquired simultaneously for both the donor and the acceptor. The sample was not bleached to 100% to avoid oxidation of the sample and bleaching of the donor. Donor and acceptor fluorescence intensity in the bleached window were quantified before and after bleaching of the acceptor. Percentage FRET and bleach efficiency were calculated from donor or acceptor fluorescence intensity measured before and after photobleaching of the acceptor according to the equation $[\%FRET=(D_{after}-D_{before})/D_{after}]$ where D_{before} and D_{after} represent the emitted intensity of the donor or acceptor fluorescence collected specifically in the bleach region before and after the photobleaching, respectively (Kenworthy, 2001; Wouters et al., 2001).

Fluorescence plate reader assay

NIH-3T3 cells were plated at a density of 5000 cells per well on 96-well plates and cell-containing wells and wells coated with soluble FN at various concentration were fixed with 3% parafomaldehyde, rinsed extensively and labeled with anti-FN and Alexa488 anti-rabbit secondary antibodies to determine the concentration (20 μg ml^{-1}) of soluble FN that generated an equivalent signal to that of cell associated FN fibrils. Subsequently, empty wells, NIH-3T3 cell containing wells and wells coated with 20 μg ml^{-1} FN or BSA were incubated in parallel with 25 μg ml^{-1} PGI/AMF-FITC for 30 minutes at pH 7.5 or pH 5.0, rinsed, fixed and then labeled with anti-FITC and Alexa488 anti-rabbit secondary antibodies. Fluorescence intensity of the labeled wells was measured using a Bio-Tek FL600 fluorescence plate reader.

Wound healing motility assay

$FN^{+/-}$ and $FN^{-/-}$ fibroblasts were plated on 35 mm plastic dishes for 2 days at 37°C in a humidified 5% CO_2 95% air incubator in complete DMEM until confluency. The monolayer was wounded by scraping from the middle of the plate followed by incubation of the cells in regular medium supplemented with 25 μg ml^{-1} PGI/AMF, as indicated, for 14 hours or for 30 minutes at pH 7.5 or pH 5 and then washed and incubated in regular medium for 13.5 hours. In some cases, the cells were pretreated with 10 μg ml^{-1} HS for 60 minutes at neutral pH before addition of PGI/AMF at pH 5 for 30 minutes. Cells were fixed and images collected with a 10× objective of an Olympus IX71 microscope. Images were analysed and cell motility quantified with ImagePro image analysis software by measuring the distance from the scrape of the 10 most motile cells for 4 fields of each condition.

Results

Direct interaction of PGI/AMF with FN at acid but not neutral pH

To determine the specificity of PGI/AMF-FN interaction, we studied PGI/AMF endocytosis and recycling in $FN^{-/-}$ fibroblasts. Compared with heterozygous $FN^{+/-}$ fibroblasts, FN homozygous mutated $FN^{-/-}$ fibroblasts do not express FN fibrils on the cell surface (Fig. 1). Some FN did associate with the cells, apparently from FN present in the serum, but did not form fibrils. Following a 30 minute pulse with PGI/AMF conjugated with Alexa 568 (PGI/AMF-568), internalized PGI/AMF-568 is localized to punctate vesicular structures corresponding to multivesicular bodies (MVBs) (Le et al., 2000) in both cell types. However, a fibrillar distribution of PGI/AMF was observed only in the $FN^{+/-}$ cells where it co-localized with cell surface FN fibrils (Fig. 1A-F). Similarly,

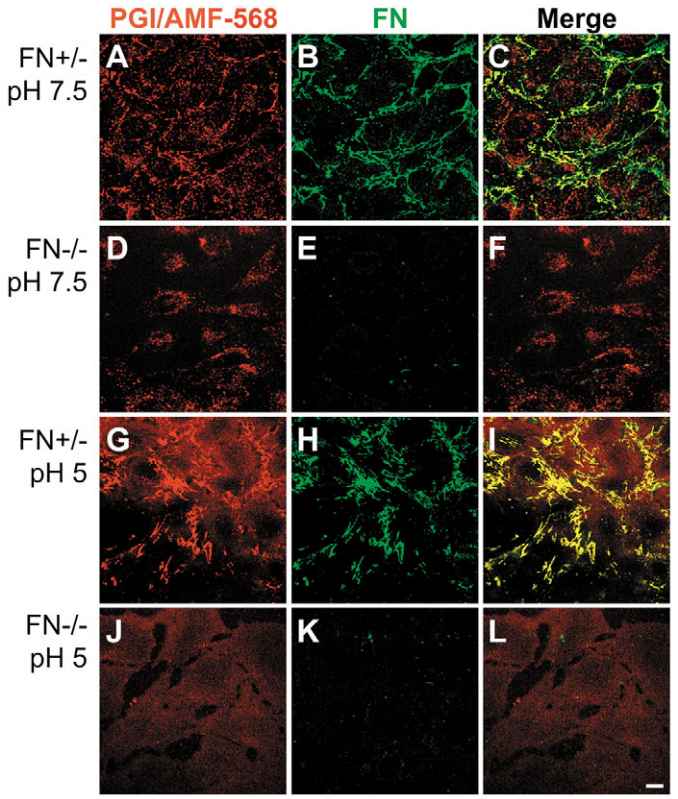


Fig. 1. PGI/AMF binding to cell surface fibrils requires FN expression. $FN^{+/-}$ (A-C,G-I) and $FN^{-/-}$ (D-F,J-L) fibroblasts were plated for 2 days on glass cover slips. The cells were incubated with 25 μg ml^{-1} PGI/AMF conjugated to Alexa-568 (PGI/AMF-568) for 30 minutes at 37°C in medium buffered to pH 7.5 with Hepes (A-F) or to pH 5.0 with MES (G-L) and then fixed with 3% paraformaldehyde. PGI/AMF-568 labeling is shown in red (A,D,G,J) and FN labeled with mouse anti-FN mAb and Alexa-488 conjugated anti-mouse secondary antibody shown in green (B,E,H,K). Merged images show co-localization of the two in yellow (C,F,I,L). Bar, 10 μm.

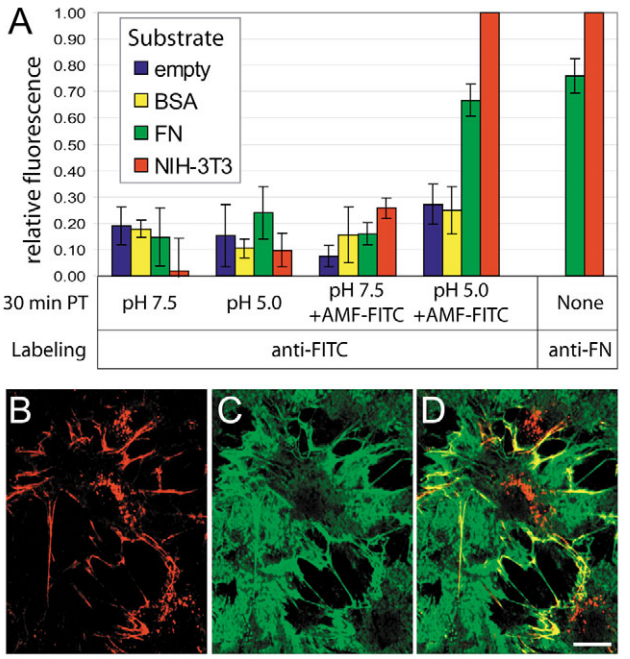
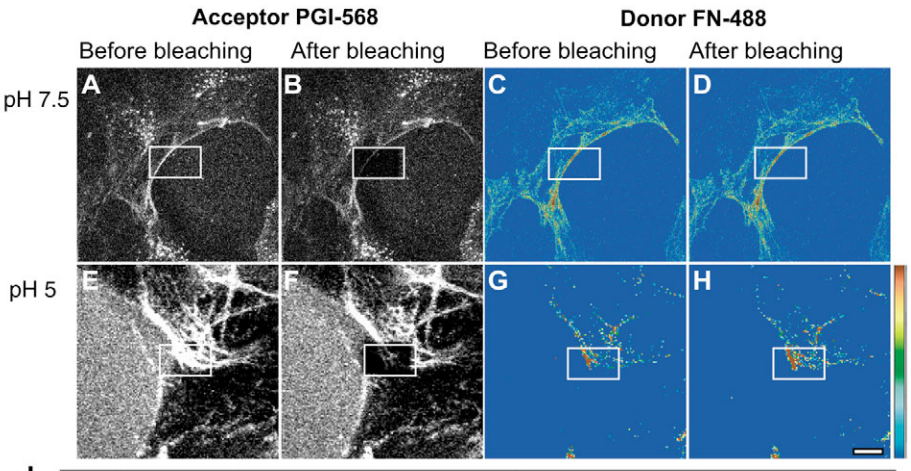


Fig. 2. Binding of PGI/AMF to dimeric FN at neutral and acid pH. The fluorescent signal due to binding of PGI/AMF-FITC to uncoated wells of a 96-well plate, to wells coated with 20 μg ml^{-1} BSA or FN, or to wells plated with NIH-3T3 cells was amplified by anti-FITC and Alexa488 anti-rabbit secondary antibodies and measured with a fluorescence plate reader (A). Absolute relative fluorescence values were normalized to maximal values and binding at pH 5 and pH 7.5 in the presence or absence of PGI/AMF-FITC was determined. To assess relative FN levels in the wells containing soluble FN or NIH-3T3 cells, parallel wells were labeled with anti-FN and Alexa488 anti-rabbit secondary antibodies (±s.e.m., n=4). Alternatively, NIH-3T3 cells were plated for 2 days on cover slips coated with 20 μg ml^{-1} of FN. The cells were then incubated with 25 μg ml^{-1} bPGI/AMF for 60 minutes at 37°C. After fixation with 3% paraformaldehyde, bPGI/AMF was revealed with Texas Red-streptavidin (B) and FN labeled with mouse anti-FN mAb and Alexa-488 conjugated anti-mouse secondary antibody (C). bPGI/AMF appears in red and FN in green and co-localization of the two appears in yellow in the merged image (D). PGI/AMF binds selectively to the fibrillar form of FN in NIH-3T3 cells plated on a FN substrate. Bar, 20 μm.

PGI/AMF exhibited increased association with FN fibrils of $FN^{+/-}$ cells at pH 5 (Fig. 1G-I). However, for $FN^{-/-}$ cells, endocytosis is blocked by acid pH and a fibrillar association of PGI/AMF was not observed (Fig. 1J-L). The cell surface fibrillar association of PGI/AMF is therefore highly specific for FN expression at neutral and acid pH.

We subsequently determined whether PGI/AMF binds to soluble FN using a fluorescent plate reader assay. To compare PGI/AMF labeling to soluble FN with that of cell associated FN, a soluble FN concentration (20 μg ml^{-1}) that generated an equivalent fluorescent signal using anti-FN antibody relative to plated NIH-3T3 cells was determined (Fig. 2A). At pH 7.5, PGI/AMF-FITC did not exhibit detectably increased binding to soluble FN or to NIH-3T3 cells relative to control wells left empty or coated with an equivalent concentration of BSA. Binding to FN at pH 5 was detectable and was essentially equivalent to PGI/AMF binding to NIH-3T3 cells (Fig. 2A) indicating that at acid pH, PGI/AMF binds to soluble FN. Due to the limited sensitivity of the assay, we were unable to detect PGI/AMF-FITC binding at pH 7.5 and therefore assessed whether at neutral pH PGI/AMF selectively interacts with fibrillar forms of FN by immunofluorescence microscopy of NIH-3T3 cells on FN coated cover slips. After 48 hours culture, both an elaborate fibrillar network of FN as well as substrate-associated FN are visualized (Fig. 2C). After incubation with 25 μg ml^{-1} of bPGI/AMF for 60 minutes at pH 7.5, confocal images show that bPGI/AMF is incorporated into MVBs and co-localizes preferentially with FN fibrils and not the non-fibrillar FN substrate (Fig. 2B-D).

To determine the nature of the association between PGI/AMF and FN at both neutral and acid pH, we performed FRET analysis by acceptor photobleaching of NIH-3T3 cells incubated for 60 minutes with PGI/AMF-568 and FN-488 at either pH 5 or pH 7.5 (Fig. 3). While a FRET efficiency of ~14.6±1.5% was detected for interaction between PGI/AMF-568 and FN-488 at pH 5, essentially no energy transfer was detected at pH 7.5. The efficiency of transfer between PGI/AMF-568 and FN-488 at pH 5 was significant and



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pH	Donor	Acceptor	Number of Cells	%Bleach ± SEM	%FRET ± SEM (** p<0.01)
7.5	FN-488	PGI-568	29	77.5 ± 1.2	1.02 ± 0.8
7.5		PGI-568	15	69.2 ± 3.5	0.22 ± 1.6 ns
7.5	FN-488		22	0.56 ± 2.9	1.56 ± 0.4 ns
7.5	FN-488	FN-568	19	71.4 ± 2.2	11.1 ± 1.4 **
5	FN-488	PGI-568	30	69.2 ± 2.8	14.6 ± 1.5 **
5		PGI-568	8	67.9 ± 3.1	-0.6 ± 2.8 ns
5	FN-488		6	2.6 ± 2.1	2.1 ± 3.8 ns

Fig. 3. FRET analysis shows the direct interaction of PGI/AMF and FN at pH 5 but not at pH 7.5. NIH-3T3 cells incubated with FN-488 for 30 minutes at pH 7.5 in complete HEPES-buffered medium were then incubated with PGI/AMF-568 for 30 minutes at 37°C at either pH 7.5 in complete HEPES-buffered medium (A-D) or at pH 5 in complete MES-buffered medium (E-H), washed with PBS/CM, fixed with 3% paraformaldehyde and the cover slips mounted in Airvol for confocal photobleaching FRET analysis. Single scan images before bleaching of the acceptor PGI/AMF-568 (A,E) and donor FN-488 (C,G) were collected and then the acceptor was bleached to 60% to 90% in the zone of interest (indicated by the box). New single scan images were then acquired simultaneously for both the donor (B,F) and the acceptor (D,H). Donor images are presented in pseudocolor (see bar to right) to highlight increased donor fluorescence after acceptor photobleaching. The extent of bleaching (% bleach) and increased donor fluorescence (% FRET) in the bleached region was quantified and is presented in table form (I) for FN-488 and PGI/AMF-568 as donor/acceptor pairs at both pH 5 and 7.5. Negative controls include donor alone or acceptor alone at pH 7.5 or 5.0 and as a positive control FRET between FN-488 and FN-568 was measured, as indicated. *P* values were determined by ANOVA relative to the FN-488 and PGI/AMF-568 pair at pH 7.5.

corresponded to that obtained between FN-568 and FN-488. No transfer was observed in the absence of acceptor or donor indicating that the energy transfer is specific. The Föster radius for Alexa488 and Alexa568, defining the distance at which energy transfer is 50% efficient, is 62 Å (Molecular Probes website). At pH 5, PGI/AMF and FN are therefore in close proximity and directly interacting.

PGI/AMF endocytosis is necessary for its association with FN fibrils on the cell surface

We undertook to determine the extent to which PGI/AMF association with FN fibrils requires prior receptor-mediated PGI/AMF endocytosis and recycling. NIH-3T3 cells were infected with an adenovirus expressing wild type dynamin-1 (dynWT), the dominant negative form of dynamin-1 (dynK44A) that blocks both clathrin-dependent and the

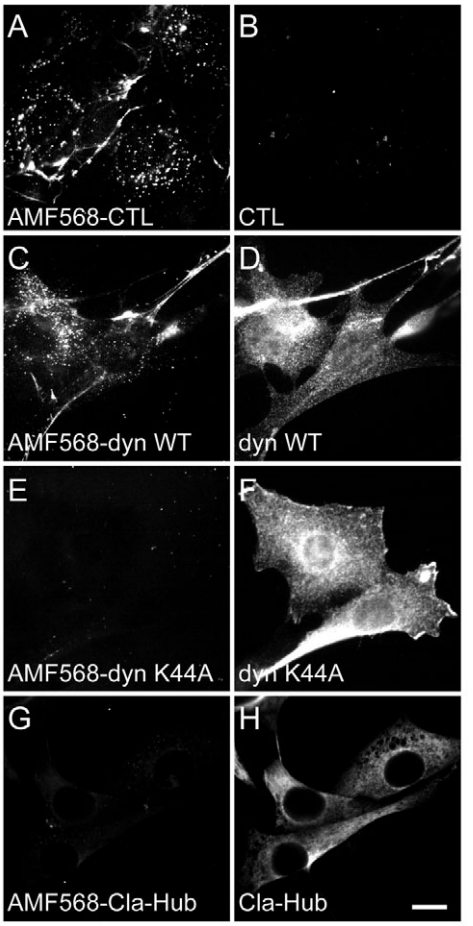

Fig. 4. PGI/AMF endocytosis is required for its association with cell surface FN fibrils. NIH-3T3 cells were uninfected (A,B) or infected for 48 hours with adenoviruses expressing wild-type dynamin-1 (dynWT) (C,D), the dominant negative dynamin-1 K44A mutant (dynK44A) (E,F) and the dominant negative clathrin hub (Cla-Hub) (G,H). The cells were then incubated with 25 μg ml^{-1} of PGI/AMF-568 for 60 minutes (A,C,E,G) and fixed with 3% paraformaldehyde. Infection rates were from 50-80% and in the selected fields, all cells were infected. Uninfected, dynWT and dynK44A infected cells were labeling with mouse anti-HA antibody and goat Alexa-488 anti-mouse secondary antibody to identify infected cells (B,D,F) and clathrin hub infected cells were identified by labeling with mouse anti-T7 antibody followed by labeling with goat Alexa-488 anti-mouse secondary antibody (H). Bar, 20 μm.

caveolae/raft-dependent endocytosis, or the clathrin hub that blocks specifically clathrin-dependent endocytosis (Altschuler et al., 1998; Altschuler et al., 1999; Le and Nabi, 2003). Infection with dynWT does not affect the endocytosis and recycling of PGI/AMF-568 (Fig. 4A-D) however infection with dynK44A or the clathrin hub prevents both PGI/AMF uptake to MVBs and its association with cell surface fibrils (Fig. 4E-H). To ensure that the reduced fibril association of PGI/AMF in the infected cells was not due to decreased FN fibrillogenesis, parallel cultures were incubated with PGI/AMF-568 and then labeled for FN. DynK44A and clathrin hub infected cells that presented reduced endocytosis of PGI/AMF-568 still express FN fibrils (data not shown) indicating that the clathrin-dependent endocytosis of PGI/AMF and recycling to the plasma membrane is a prerequisite for its association with FN fibrils.

Co-incubation of cells with biotinylated PGI/AMF (bPGI/AMF) and FN-FITC allowed us to segregate PGI/AMF internalization from FN fibrillogenesis. After 10 minutes of co-incubation, bPGI/AMF is localized essentially exclusively to MVBs, as previously reported (Le et al., 2000), while the vast majority of cell-associated FN is incorporated into fibrils (Fig. 5A-C). After 60 minutes co-incubation, bPGI/AMF is localized to MVBs as well as to cell surface FN fibrils (Fig. 5D-F). The minimal presence of FN in PGI/AMF-positive MVBs argues that interaction between bPGI/AMF and FN occurs following delivery of PGI/AMF to the cell surface.

If this were the case, we should be able to capture recycling PGI/AMF at the cell surface. NIH-3T3 cells were pulse labeled with bPGI/AMF for 30 minutes and, after a 10 minute wash, incubated with Texas Red streptavidin at either 37°C or 4°C for a further 30 minutes (Fig. 6). When added to cells at 37°C, bPGI/AMF was internalized to intracellular vesicular structures that co-localized with bPGI/AMF labelled after fixation with FITC-streptavidin (Fig. 6A-C). However, following incubation at 4°C, Texas Red streptavidin added to viable cells was observed to bind only to cell surface fibrillar structures (Fig. 6D-F). In the absence of a prior bPGI/AMF pulse, Texas Red streptavidin was not observed to label the cells (Fig. 6G-I). bPGI/AMF that has recycled to the plasma membrane can therefore undergo another round of endocytosis.

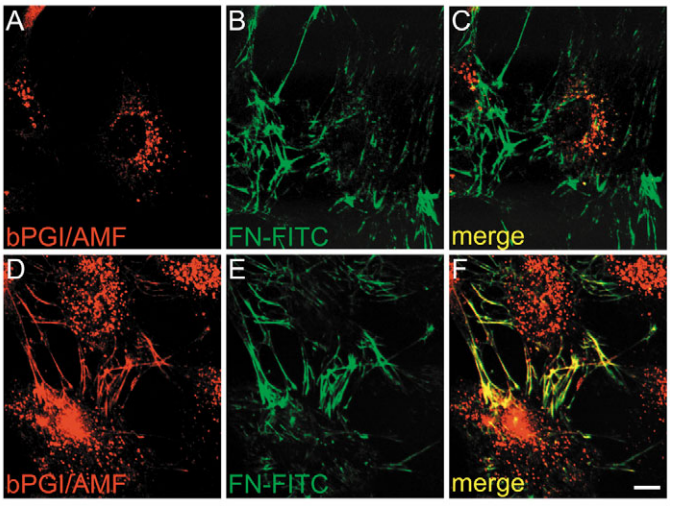

Fig. 5. PGI/AMF and FN do not co-internalize and interact at the cell surface. NIH-3T3 cells were co-incubated with 25 μg ml^{-1} bPGI/AMF and 10 μg ml^{-1} FN-FITC for 10 (A-C) or 60 (D-F) minutes at 37°C. The cells were then fixed with 3% paraformaldehyde and bPGI/AMF was revealed with Texas Red-streptavidin (A,D) and FN with anti-FN mAb and Alexa-488 conjugated secondary antibodies (B,E). Colocalization of the two appears in yellow in the merged images (C,F). Bar, 10 μm.

Acid-dependent sequestration of PGI/AMF cytokine activity by FN

HS is implicated in ECM-mediated sequestration of multiple cytokines (Vlodavsky et al., 1996) and has been shown to bind specifically to matricryptic sites in FN (Homandberg et al., 1985). To determine if HS is involved in the association of PGI/AMF with FN fibrils, NIH-3T3 cells were pretreated with

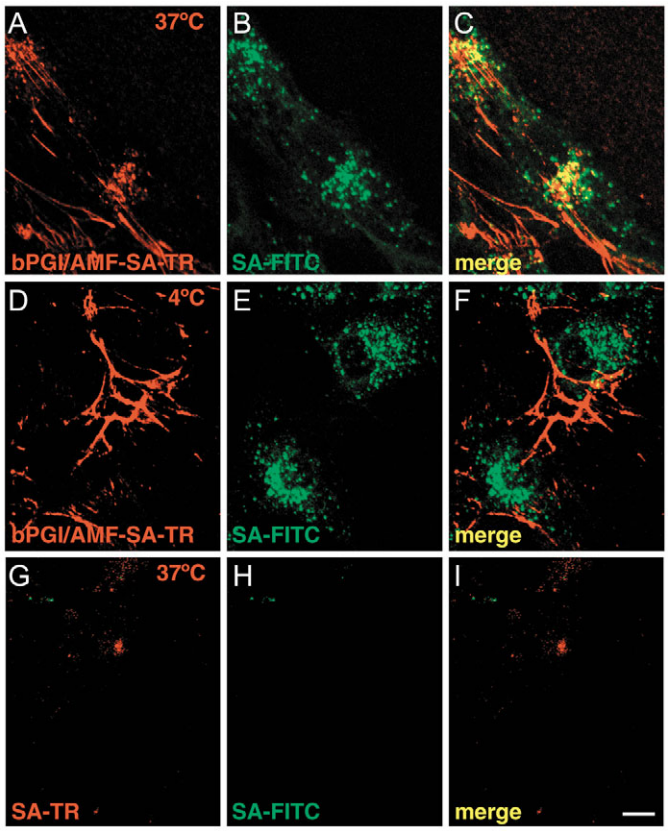

Fig. 6. Endocytosed bPGI/AMF can undergo more than one cycle of endocytosis. NIH-3T3 cells were incubated with 25 μg ml^{-1} bPGI/AMF in complete medium (A-F) or with complete medium in the absence of bPGI/AMF (G-I) for 30 minutes at 37°C. The cells were then washed three times with complete medium and then incubated 30 minutes with Texas Red-streptavidin (SA-TR) at 37°C (A-C, G-I) or at 4°C (D-F). Cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and labeled with FITC-streptavidin (SA-FITC) to detect endocytosed bPGI/AMF that had not been bound by Texas Red-streptavidin (B,E,H). Texas Red (red) and FITC-streptavidin (green) confocal images were merged and confocal co-localization appears in yellow (C,F,I). When added at 37°C (A-C) but not at 4°C (D-F), Texas Red-streptavidin can be seen to label PGI/AMF positive MVBs and was therefore captured for a second round of endocytosis by recycling bPGI/AMF. Bar, 10 μm.

various concentrations of HS (1-100 μg ml^{-1}) and then incubated with PGI/AMF-568. At neutral pH, the addition of HS did not affect the total cellular association of PGI/AMF across a range of HS concentrations (Fig. 7A). Specific association of PGI/AMF with FN fibrils was not affected by the presence of 10 μg ml^{-1} HS and pre-incubation with HS did not influence PGI/AMF endocytosis to MVBs (Fig. 7B). However, when NIH-3T3 cells pretreated with HS (at pH 7.5) were incubated with PGI/AMF-568 at pH 5.0, the cellular association of PGI/AMF was increased in a dose-dependent manner with maximal binding at 10 μg ml^{-1} HS (Fig. 7A). The increased cellular binding of PGI/AMF in the presence of HS could be competed with an excess of PGI/AMF indicating that this interaction is saturable (Fig. 7C). Quantification of the overlap of PGI/AMF labeling with FN fibrils showed that specific interaction with FN fibrils was only slightly increased and not competed by excess PGI/AMF (Fig. 7C). In the presence of HS, PGI/AMF was seen to exhibit a punctate distribution that did not co-localize with FN or FN fibrils (Fig. 7C). In FN$^{-/-}$ cells, HS still stimulated the cell surface association of PGI/AMF-568 at acid pH and the HS-dependent cell surface binding of PGI/AMF was not fibrillar but rather punctate in nature (Fig. 7D). HS therefore mediates an alternate, FN-independent mechanism for the cell surface association of PGI/AMF at acid pH.

The identification of two mechanisms for PGI/AMF cellular interaction at acid pH led us to determine whether acid-dependent sequestration of PGI could promote cell motility upon pH neutralization. Using a wound healing assay, FN$^{-/-}$ cells were observed to migrate more slowly than FN$^{+/-}$ cells and both cell types responded to the addition of PGI/AMF over a 14 hour period by exhibiting increased motility while pretreatment with HS for 60 minutes did not affect cell motility (Fig. 8). We subsequently treated the cells after wounding for 30 minutes at pH 7.5 or at pH 5 with PGI/AMF, washed the cells and determined the impact of the treatment on migration over a subsequent 13.5 hour period in regular medium. A 30 minute treatment with PGI/AMF at pH 7.5 did not affect cell

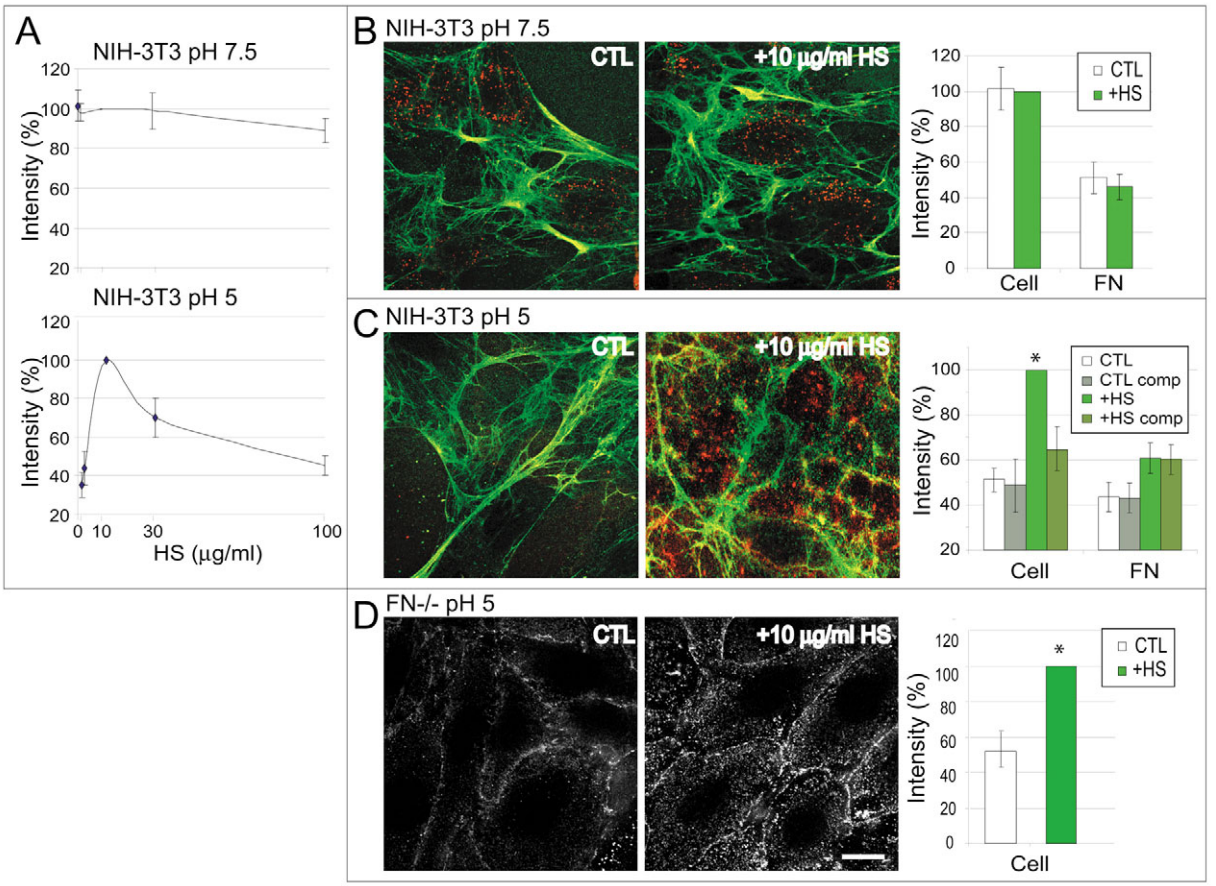

Fig. 7. Heparan sulphate stimulates FN-independent binding of PGI/AMF at pH 5. (A) NIH-3T3 cells were left untreated or treated with 1, 10, 30 and 100 μg ml^{-1} HS for 30 minutes at 37°C and then incubated for 30 minutes at 37°C with 25 μg ml^{-1} PGI/AMF-568 in HEPES adjusted medium at pH 7.5 or 5 μg ml^{-1} PGI/AMF-568 in MES adjusted medium at pH 5. Representative images show PGI/AMF-568 labeling in red and FN labeling in green in NIH-3T3 cells at pH 7.5 (B) or pH 5 (C) and PGI/AMF-568 labeling in FN$^{-/-}$ cells (D) in control cells and cells treated with 10 μg ml^{-1} HS. Bar, 20 μm. The bar graphs in B, C and D present the quantification of the fluorescent intensity of total cell-associated (Cell) and FN fibril-associated (FN) PGI/AMF-568 in the absence (CTL; white bars) or following pretreatment with 10 μg ml^{-1} HS (+HS; green bars). In NIH-3T3 cells at pH 5 (C), PGI/AMF-568 binding was competed for by incubating the cells with 100 μg ml^{-1} of unlabelled PGI/AMF before addition of 5 μg ml^{-1} PGI/AMF-568 in complete medium adjusted to pH 5 in the absence (CTL comp; grey bars) or presence of 10 μg ml^{-1} HS (HS comp; dark green). PGI/AMF-568 fluorescent intensity was quantified from 10 random images per condition and the data normalized to the condition presenting the maximum intensity (±s.e.m.; n=3; P<0.01).

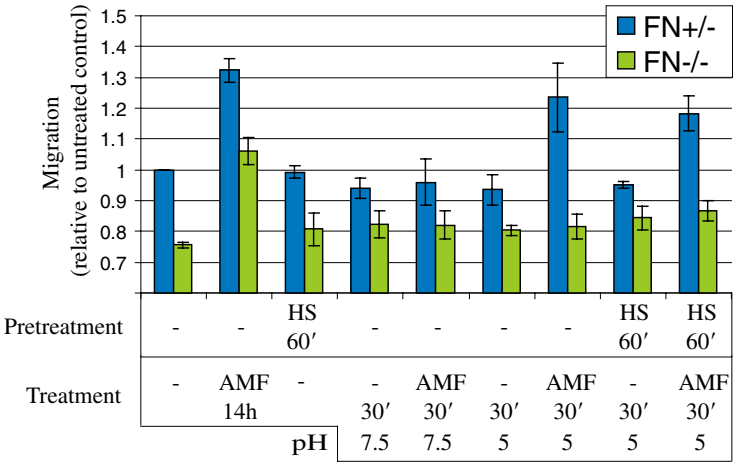

Fig. 8. Acid-dependent sequestration of PGI/AMF by FN stimulates cell motility. Confluent monolayers of $FN^{+/-}$ (blue bars) and $FN^{-/-}$ (green bars) cells were grown for 1 day in regular medium, wounded by scraping and cell migration from the wound measured after 14 hours. The scraped monolayers were treated with 25 μg ml^{-1} PGI/AMF for the complete 14 hour period or treated for 30 minutes with the same concentration of PGI/AMF at pH 5 or 7.5 and then rinsed and incubated in regular medium for the remainder of the 14 hour period. Alternatively, cells were pre-incubated with 10 μg ml^{-1} HS for 60 minutes and then incubated with HS or HS plus PGI/AMF for 30 minutes at pH 5 prior to rinsing. The data was normalized to the migration of FN+/– cells in the absence of PGI/AMF or HS (±s.e.m.; n=3).

motility however addition of PGI/AMF at pH 5 selectively enhanced the migration of $FN^{+/-}$ but not $FN^{-/-}$ cells. Preincubation with HS and subsequent addition of PGI/AMF stimulated only the migration of $FN^{+/-}$ but not of $FN^{-/-}$ cells. The acid-dependent sequestration of PGI/AMF by FN but not HS therefore promotes cell motility upon pH neutralization.

Discussion

Trafficking and FN interaction of PGI/AMF

PGI/AMF activation of its receptor activates a pertussis toxin sensitive G-protein mediated pathway and downstream protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3 kinase), MAP kinase kinase (MEK) and JUN kinase (JNK) signaling pathways as well as the small GTPases rac and rho (Nabi et al., 1990; Torimura et al., 2001; Tsutsumi et al., 2002). PGI/AMF stimulation induces the rearrangement of focal adhesion proteins and induction of stress fibers as well as the translocation of β1 integrins to the cell surface (Timar et al., 1996; Torimura et al., 2001). PGI/AMF stimulates cell adhesion and spreading of metastatic murine melanoma cells (B16a) on a FN substrate (Timar et al., 1996) as well as the incorporation of substrate FN into fibrils in low metastatic K1735-Cl11 murine melanoma cells and its degradation by high metastatic K1735–M1 cells (Silletti et al., 1998). AMFR activation of downstream effectors, including rho-mediated contractility (Tsutsumi et al., 2002; Zhong et al., 1998), is therefore implicated in ECM reorganization during cell motility.

PGI/AMF is internalized by its receptor via both clathrin- and caveolae/raft-dependent pathways (Benlimame et al., 1998; Le et al., 2000; Le et al., 2002; Le and Nabi, 2003). The clathrin-dependent pathway targets MVBs and we have previously shown that internalized PGI/AMF can recycle to cell surface FN fibrils (Le et al., 2000). The ability of adenoviral infection with both the clathrin hub and dynamin-1 K44A to prevent the association of PGI/AMF with cell surface fibrils (Fig. 4) shows that clathrin-dependent endocytosis and recycling of PGI/AMF is a prerequisite for its association with FN fibrils. PGI/AMF is internalized via both clathrin- and caveolae/raft-dependent pathways (Fig. 9) and these data are consistent with the fact that inhibition of caveolae/raft-dependent endocytosis with methyl-β-cyclodextrin does not prevent fibril association of PGI/AMF (Le et al., 2000). Co-incubation of cells with both PGI/AMF and FN shows clearly that after only 10 minutes FN is incorporated into fibrils while PGI/AMF is endocytosed to endosomes (Fig. 5). Little to no FN could be detected in PGI/AMF positive MVBs and it is therefore unlikely that this recycling pathway is involved in FN fibrillogenesis.

Many proteins interact with FN including multiple integrins as well as ECM proteins such as collagen, fibrin, thrombospondin, tissue transglutaminase and proteoglycans. FN undergoes dramatic structural changes upon fibril formation that reveal formerly cryptic protein binding sites (Geiger et al., 2001; Pankov and Yamada, 2002). Fibrinogen, thrombospondin-1 and transglutaminase associate specifically with FN fibrils and their incorporation into the ECM is further associated with fibril assembly (Dardik and Lahav, 1999; Pereira et al., 2002; Verderio et al., 1998). PGI/AMF interaction with FN at pH 7.5 is saturable and AMFR associates with FN fibrils in NIH-3T3 cells (Le et al., 2000). Our inability to detect FRET between PGI/AMF and FN (Fig. 3) and the required role for receptor recycling in PGI/AMF association with FN (Fig. 4) at neutral pH are consistent with the AMFR-mediated interaction of PGI/AMF with FN. Intraendosomal pH can acidify to pH 5.0-5.5 and receptor recycling from endosomes is regulated by endosomal pH (Marshansky et al., 2002; Tycko and Maxfield, 1982; Yamashiro and Maxfield, 1984) such that acid-induced changes of PGI/AMF conformation within the MVBs could conceivably be involved in its interaction with FN upon recycling to the cell surface. However, disruption of the endosomal pH gradient with chloroquine was not observed to affect PGI/AMF recycling to FN fibrils (data not shown). Distinct mechanisms therefore mediate the interaction of PGI/AMF and FN at neutral and acid pH.

The demonstration that PGI/AMF can recycle from MVBs for more than one cycle argues that upon recycling to the cell surface, the PGI/AMF-AMFR complex does not interact directly with FN but can be reinternalized (Fig. 9). Plating cells on a PGI/AMF-coated substrate inhibited cell motility and the clathrin- but not the caveolae/raft-dependent uptake of PGI/AMF (Le et al., 2000) implicating the clathrin-dependent PGI/AMF-AMFR recycling pathway in the regulation of AMFR signaling in cell motility. Reduced FN expression has

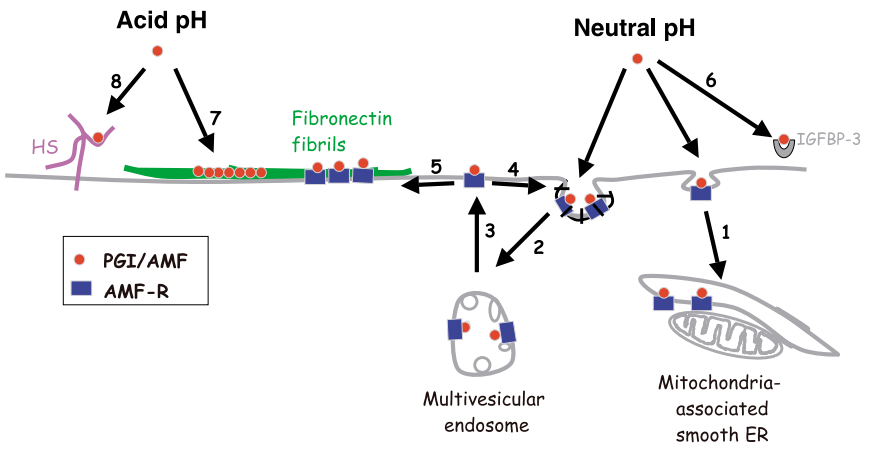

Fig. 9. The complex biology of PGI/AMF and its receptor. At neutral pH, PGI/AMF binds its receptor AMFR at the cell surface and can be endocytosed via two different pathways: caveolae/raft-dependent endocytosis to the smooth ER (1) or clathrin-dependent endocytosis to multivesicular bodies (MVBs) (2). Internalized PGI/AMF can recycle from MVBs to the plasma membrane (3) where it can undergo further rounds of endocytosis and recycling (4). Recycling receptor-ligand complexes can also be sequestered via stable association with FN fibrils (5). PGI/AMF can also interact directly with IGFBP-3 (6), an interaction that reduces its cellular binding. At acid pH, endocytosis is inhibited and PGI/AMF binds directly to FN fibrils (7) or to heparan sulphate (HS) (8).

long been associated with transformation and tumor progression (Hynes and Wyke, 1975; Olden and Yamada, 1977) and polymeric or superfibronectin inhibits tumor growth and metastasis (Pasqualini et al., 1996; Yi and Ruoslahti, 2001). Sequestration of recycling PGI/AMF-AMFR complexes by association with FN fibrils may regulate the extent of ligand-dependent AMFR signaling.

Acid-dependent sequestration of PGI/AMF

PGI/AMF is an oligomeric protein whose enzymatic function is dependent on dimer formation (Bruch et al., 1976) and acidification to pH 5 is associated with loss of enzymatic activity due to disruption of PGI/AMF oligomeric structure (Amraei et al., 2003; Dyson and Noltmann, 1968). We have previously shown that the acid-induced association of PGI/AMF with FN is associated with both changes in the tertiary structure of the protein and disruption of the oligomeric structure of the protein (Amraei et al., 2003). The detection of FRET between PGI/AMF and FN at pH 5 indicates that interaction between the proteins is direct and supports a role for the altered conformation of PGI/AMF at acid pH as a mediator of its interaction with FN. While intramolecular FRET has been described for FN (Baneyx et al., 2001; Baneyx et al., 2002), we are not aware of any studies showing FRET between FN and a FN-binding protein. Intermolecular FRET between FN and PGI/AMF at pH 5 therefore demonstrates clearly the direct protein-protein interaction between FN and a cytokine.

The role of heparan sulphate proteoglycans in the sequestration by the ECM of angiogenic factors, such as VEGF, FGF2 and TGF-β, chemokines, such as interleukin-8 and platelet factor 4, and growth factors, including IGF-I and TGF-β, is well established (Folkman and Shing, 1992; Ruoslahti et al., 1992; Vlodavsky et al., 1996). Heparin-mediated interaction with the ECM protects cytokines from degradation by matrix metalloproteases and concentrates cytokines for subsequent release upon matrix degradation (Miao et al., 1996). HS selectively increases cellular PGI/AMF binding at acid and not neutral pH identifying HS as a cellular receptor for PGI/AMF under acidic conditions. Although FN has two defined HS-binding sites (Homandberg et al., 1985), the presence of HS does not enhance PGI/AMF binding to FN fibrils and increases PGI/AMF binding to $FN^{-/-}$ cells. HS therefore mediates the FN-independent interaction of PGI/AMF with the cell under acidic conditions. Acid-induced conformational changes of PGI/AMF (Amraei et al., 2003) therefore regulate both its binding to FN and to HS. Similarly, calcium-dependent conformational changes in thrombospondin mediate its interaction with both FN and heparin (Dardik and Lahav, 1999).

We have therefore identified two acid-specific mechanisms for the sequestration of PGI/AMF: direct interaction with FN and FN-independent binding to cell surface-associated HS. Recently, insulin-like growth factor binding protein-3 (IGFBP-3) was shown to interact with cell surface PGI/AMF and inhibit both its cellular binding and its cytokine activity (Mishra et al., 2004). Acid-dependent sequestration of PGI/AMF by FN is shown here to stimulate cell motility upon pH neutralization. By contrast, sequestration of PGI/AMF by receptor-mediated recycling to FN fibrils at neutral pH or by HS at acid pH is not associated with enhanced motility (Fig. 8). The amount of additional PGI/AMF that is cell-associated in the presence of HS at acid pH is of the same order as that bound by FN (Fig. 7C) and why PGI/AMF sequestered at acid pH by FN and not HS can be released in an active form to stimulate cell motility is not clear. PGI/AMF sequestered by FN at acid pH can subsequently be internalized to MVBs upon pH neutralization (Amraei et al., 2003) and release from FN may facilitate its subsequent receptor-mediated endocytosis via the clathrin pathway that promotes cell motility. Alternatively, interaction with FN may serve to concentrate PGI/AMF monomers in such a way that upon pH neutralization active dimers are released.

The extracellular microenvironment of tumors presents variable pH ranging from 5.6-7.6 (Vaupel et al., 1989). Ischemia is also associated with reduced interstitial pH and has been measured as low as pH 5.5 in the ischemic cortex of hyperglycemic rat brain (Nedergaard et al., 1991). Surface pH measurements of osteoarthritic cells reported local pH values of 5.5 suggesting that local pH values may be quite acidic (Konttinen et al., 2002). While binding of PGI/AMF to FN is strongest at pH 5, interaction with FN was also observed at pH 5.5 and to a lesser extent at pH 6 (Amraei et al., 2003). Hypoxia is associated with local acidification and results in the activation of hypoxia inducible factor 1 (HIF-1), a transcription factor that induces transcription of genes coding for glycolytic enzymes and angiogenic factors (Dang and Semenza, 1999), including PGI/AMF (Niizeki et al., 2002; Yoon et al., 2001).

Increased expression of glycolytic enzymes and glycolysis under hypoxic conditions would therefore result in the increased secretion of PGI/AMF into an acidic milieu. The specific sequestration of PGI/AMF and other angiogenic cytokines, such as VEGF (Goerges and Nugent, 2003; Goerges and Nugent, 2004), under acidic conditions may serve to concentrate proangiogenic factors at sites of tissue damage to promote angiogenesis towards the damaged region upon resolution of the tissue insult.

Multiple pH selective mechanisms therefore exist for the cell surface interaction of PGI/AMF. The cytokine function of PGI/AMF is specific for the mammalian and not the bacterial or yeast forms of this conserved glycolytic enzyme and was evidently acquired evolutionarily (Amraei and Nabi, 2002). The PGI/AMF receptor, AMFR, is a ubiquitin E3 ligase that contains RING and CUE domains (Fang et al., 2001; Shimizu et al., 1999). A database search for CUE domain-containing proteins identified only a *Caenorhabditis elegans* homolog for AMFR and metazoan AMFR was proposed to result from the fusion of a yeast RING-finger-containing protein and Cue1p, a ubiquitin scaffolding protein (Ponting, 2000). Acquisition of PGI cytokine function and expression of its receptor may have occurred coordinately early during the evolution of multicellular organisms. The multiple cell surface interactions of this ligand and elaborate trafficking of its receptor (Fig. 9) may represent acquired mechanisms to regulate and harness the cytokine function of this ubiquitous, circulating cellular protein.

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