Insulin-Like Growth Factor Binding Protein-3 Interacts with Autocrine Motility Factor/Phosphoglucose Isomerase (AMF/PGI) and Inhibits the AMF/PGI Function

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ABSTRACT

Autocrine motility factor/phosphoglucose isomerase (AMF/PGI) was identified as a binding partner for insulin-like growth factor binding protein-3 (IGFBP-3) in solubilized T47D and MCF-7 human breast cancer cells. The interaction between AMF/PGI and IGFBP-3 was verified by cross-linking biotinylated IGFBP-3 to intact cells. After solubilization of the membranes, the biotinylated complexes were precipitated with streptavidin-agarose conjugate and analyzed by SDS-PAGE. A Mr ~80,000 complex was identified when the nitrocellulose membranes were probed either with streptavidin-horseradish peroxidase conjugate or AMF/PGI antiserum confirming the cross-linking of IGFBP-3 to AMF/PGI. The interaction between IGFBP-3 and AMF/PGI was also further confirmed by ligand blotting of purified AMF/PGI using biotinylated IGFBP-3. Both glycosylated and nonglycosylated IGFBP-3 inhibited the catalytic activity of AMF/PGI in a dose-dependent fashion. In addition, IGFBP-3 inhibited the binding of AMF/PGI to breast cancer cells and AMF/PGI-induced migration of both T47D and MCF-7 human breast cancer cells. IGFBP-3 also decreased the phosphorylation of AMF/PGI and reduced the translocation of AMF/PGI to the cell membrane and AMF/PGI. AMF/PGI resulted in a dose-dependent inhibition of IGFBP-3-induced apoptosis in T47D and MCF-7 cells. In summary, we have identified AMF/PGI as a membrane-associated binding partner for IGFBP-3 in breast cancer cells. The ability of IGFBP-3 to bind and inhibit the actions of AMF/PGI may have some role in the antiproliferative proapoptotic effects of IGFBP-3.

INTRODUCTION

Insulin-like growth factor binding protein-3 (IGFBP-3) is the most abundant of the IGFBPs in the circulation and functions to transport and modulate the actions of insulin-like growth factors (IGF-I and -II). IGFBP-3 can both enhance the effects of IGF-I presumably by delivering IGF-I to its plasma membrane receptor and compete with the receptor for binding IGF-I and thereby inhibit IGF-I action (1, 2). In addition to these IGF-dependent effects, emerging evidence suggests that IGFBP-3 also functions directly to stimulate apoptosis and to inhibit cellular proliferation of various cell lines including human breast cancer cells (3–7). These direct effects are clearly demonstrable with mutant IGFBP-3 and IGFBP-3 fragments, which have minimal affinity for IGF-I (5–7), and with cell lines devoid of IGF-I receptors (3, 7). Thus, these direct effects have been termed IGF-independent. The mechanisms that mediate these IGF-independent effects are not known but probably involve the interaction with cell surface receptors (8).

Membrane binding sites for IGFBP-3 have been identified on a variety of cell lines using cross-linking agents (8–10). However, the identity of these IGFBP-3 binding sites has not been clarified, although IGFBP-3 has been shown to bind in a specific, saturable manner to the type V transforming growth factor-β receptor (11). The importance of this observation is unclear because the type V transforming growth factor-β receptor is not widely expressed and does not appear to be important in the transduction of transforming growth factor-β action in most cell lines. More importantly IGFBP-3 has proapoptotic effects in cells that do not appear to express the type V transforming growth factor-β receptor (10).

In an attempt to further understand the mechanisms underlying the IGF-independent actions of IGFBP-3, we have attempted to purify IGFBP-3 binding partners from solubilized T47D cell membranes. Here we report that IGFBP-3 is able to bind to autocrine motility factor/phosphoglucose isomerase (AMF/PGI), which is a C-X-C-X-C cytokine secreted by a variety of cell types (12). AMF/PGI, in addition to its enzymatic function, is an antiapoptotic cytokine that stimulates proliferation and migration of a variety of cells in an autocrine fashion via interaction with a Mr 78,000 glycoprotein receptor, AMF receptor (AMF-R; Ref. 13).

MATERIALS AND METHODS

Materials and Reagents. T47D and MCF-7 cells were obtained from the American Type Tissue Collection (Manassas, Virginia). Cells were cultured as described previously (14) using culture reagents were Life Technologies, Inc. (Burlington, Ontario, Canada). Glycosylated and nonglycosylated IGFBP-3 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). All other reagents were obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada) unless otherwise stated.

Biotinylation of IGFBP-3 and AMF/PGI. Non glycosylated Escherichia coli-derived IGFBP-3 was biotinylated as described previously (14). Biotinylated IGFBP-3 retained full biological activity in terms of binding to IGF-I and binding to cell membranes (14). Rabbit muscle-derived AMF/PGI (Sigma-Aldrich Canada) was dissolved in 500 µl of PBS at a concentration of 1 µg/µl and was incubated with 7.5 µl of p-biotinoyl-aminocaproic acid-N-hydroxy-succinimide ester (Roche Molecular Biochemicals, Mannheim, Germany) for 2 h at room temperature. At the end of the incubation, free biotin ester was separated on a Sephadex G-25 column equilibrated with 5 ml of blocking solution (Roche Molecular Biochemicals). The sample was eluted with PBS and collected in 0.4-ml fractions. The protein concentration was measured using Bradford protein assay (Bio-Rad Laboratories Inc. Mississauga, ON, Canada). Biotinylated AMF/PGI remained full catalytic activity (data not shown). The biotinylated samples were stored at ~80 C until used. E. coli-derived IGFBP-3 was also biotinylated in the same way as described above and was stored at ~80 C until used.

Cross-Linking of Biotinylated IGFBP-3. For cross-linking studies, biotinylated-IGFBP-3 was immobilized on streptavidin-coated tubes. Solubilized cell membranes, prepared as described previously (14), were incubated in IGFBP-3-immobilized tubes in PBS for 1 h on ice. Then disuccinimidyl suberate (DSS, 100 µg/ml) was added and the incubation continued for another 20 min. Finally-IGFBP-3-cross-linked membrane proteins were washed in ice-cold PBS, boiled in SDS sample buffer, and analyzed on 11% SDS-PAGE. Proteins were transferred to the nitrocellulose membrane and were detected with streptavidin-horseradish peroxidase (HRP) conjugate using the enhanced chemiluminescence (ECL) system. For cross-linking studies with cell mono-
layers, cells were grown to near confluence on 35-mm culture dishes. The monolayer was incubated with biotinylated-IGFBP-3 in PBS for 1 h at 4°C. DSS (100 g/ml) was added for the final 20 min. Cells were washed three times in ice-cold PBS and were recovered in ice-cold PBS containing aprotinin (2 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM EDTA. Streptavidin-agarose was used to recover cross-linked proteins, which were analyzed as above.

**Chromatography and Protein Identification.** Solubilized membrane proteins from T47D cells was dissolved in starter buffer [0.05 M sodium phosphate and 0.15 M NaCl (pH 7.2)], filtered, and loaded to Sephacyrl S-100HR (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec, Canada) column. Eluted fractions were collected as 3-ml aliquots. Alternate gel fractions in the molecular weight range of M, 20,000–80,000 were subjected to High Q anion exchange chromatography (Bio-Rad, Mississauga, Ontario, Canada). Separation was performed in a linear gradient from 0.05 to 0.5 M NaCl over 50 min at a flow rate of 1 ml/min, and 1-ml fractions were collected. Desalted and buffer-exchanged fractions containing the protein of interest were further purified using IGBP-3-Sepharose 4B affinity chromatography. Bound proteins were first eluted with 0.05 M sodium phosphate containing 0.15 M NaCl (pH 7.2) followed by 0.1 M acetic buffer containing 0.5 M NaCl (pH 4.0). Eluted fractions were desalted, concentrated, and analyzed on SDS-PAGE. Bands of interest were visualized by silver staining and ligand and immunoblotting techniques. From the silver-stained gel, bands of interest were excised and processed for matrix-assisted laser desorption/ionization mass spectrometry spectrum time of flight. Identification of the tryptic peptides was done by searching database against the peptide fingerprints using Mascot search engine (http://www.matrixscience.com).

**Immunoprecipitation.** Solubilized membranes were precipitated by anti-AMF and protein A-agarose conjugate and centrifuged, and pellet was washed three times in ice-cold PBS, boiled in loading buffer, and separated on 11% gel. Separated proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in 5% milk, washed in Tris-buffered saline with 0.5% Tween 20, and incubated with streptavidin-HRP conjugate diluted to 1:5000 for 1 h at room temperature. Immunoblotting techniques. From the silver-stained gel, bands of interest were excised and processed for matrix-assisted laser desorption/ionization mass spectrometry. Bands of interest were visualized by silver staining and ligand and immunoblotting techniques. From the silver-stained gel, bands of interest were excised and processed for matrix-assisted laser desorption/ionization mass spectrometry. Bands of interest were visualized by silver staining and ligand and immunoblotting techniques. From the silver-stained gel, bands of interest were excised and processed for matrix-assisted laser desorption/ionization mass spectrometry.
immunoprecipitated with anti-AMF antibody, resolved on 11% SDS-PAGE, and analyzed by autoradiography and by immunoblotting with anti-AMF antibody.

**Cell Motility Assay.** T47D and MCF-7 cells were used for the cell motility assays, which were performed using a modification of the method described by Talukder et al. (15). Polycarbonate filters (Costar Corp., Cambridge, MA; 8 μm pore size) were coated with collagen (20 μg/filter) and were dried overnight. Cells (10^5) were placed in top of a Boyden chamber in serum-free DMEM containing 0.1% BSA. The filter inserts with cells were placed in wells of 24-well culture plates containing 650 μl of serum-free medium containing 0.1% BSA as control, medium plus AMF (100 pg/ml), and various concentrations of IGFBP-3. Incubation was carried out at 37°C for 24 h. The filters were removed and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells on upper filter surface were removed carefully with cotton swab. The filters were stained in hematoxylin for 10 min, and cells on the lower surface of the filter were counted under a light microscope.

**Determination of AMF/PGI Enzymatic Activity.** Isomerase activity was assayed as described by Gracy and Tilley (16). The reaction was initiated by the addition of rabbit AMF/PGI (0.1 unit/ml) to 1 ml of reaction mixture [50 mM triethanolamine buffer (pH 8.3), 1 mM EDTA, 4 mM fructose 6-phosphate, 0.1% BSA] as control, medium plus AMF (100 pg/ml), and various concentrations of IGFBP-3. Incubation was carried out at 37°C for 24 h. The filters were removed and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells on upper filter surface were removed carefully with cotton swab. The filters were stained in hematoxylin for 10 min, and cells on the lower surface of the filter were counted under a light microscope.

**Equilibrium Binding Assay.** An immobilized ligand-based assay system was used to characterize the binding of biotinylated-IGFBP-3 and biotinylated-IGFBP-3:IGF-I binary complex from immobilized AMF/PGI. Rabbit AMF/PGI (250 ng/well) was coated on 96-well Maxisorp plates (Nunc, Kamstrup, Denmark) in 100 mM carbonate buffer (pH 9.4) overnight at 4°C and was subsequently blocked with 1% BSA. Plates were rinsed twice with 200 μl of PBS (pH 7.4) and once with assay buffer [30 mM Tris acetate (pH 7.4), 10 mM sodium phosphate, 0.1% Tween 20]. For binding assay, biotinylated-IGFBP-3 or biotinylated-IGFBP-3:IGF-I binary complex (10 ng/well) was incubated together with various concentrations of unlabeled IGFBP-3 or binary complex in 100 μl of assay buffer for 1 h at 37°C. Unbound ligand was removed by rinsing the wells twice with 200 μl of ice-cold assay buffer. Bound ligand was detected using streptavidin-alkaline phosphatase conjugate and p-Nitrophenyl Phosphate (1 mg/ml) as substrate. The absorbance was read on a microplate spectrophotometer at 405 nm, and the amount of bound ligand was determined by reference to a standard curve generated using serial dilution of biotinylated IGFBP-3. After the determination of free and bound ligand, the equilibrium-binding constants were determined by Scatchard analysis.

**IGFBP-Induced Apoptosis.** Apoptosis was quantified after 48-h pretreatment with 100 ng/ml IGFBP-3. IGFBP-3-induced apoptosis was determined by quantifying histone-associated DNA fragments (mono- and oligonucleosome) in cell lysate using the Cell Death ELISA kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. In brief, a 96-well plate was coated with monoclonal antihistone antibody and was subsequently blocked with blocking solution. One hundred μl of prediluted samples (1:10 with incubation buffer) were added to each well and incubated for 90 min at room temperature. After washing three times with wash buffer, 100 μl of anti-DNA-peroxidase conjugate was added to each well except blank and were incubated for 90 min at room temperature. At the end of the incubation, wells were washed as above, and 100 μl of ABTS (2, 2’-azino-di[3-ethylbenzthiazoline sulfonate]) substrate solution (1 mg/ml) were added and incubated on a plate shaker at 250 rpm. Absorbance was taken at 405 nm after 15 min.

**Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Assay.** Apoptosis in T47D and MCF-7 cells were also determined using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Cells (30,000) were plated on Lab-Tec chamber slides (Nunc Inc., Kamstrup, Denmark) in DMEM (10% fetal bovine serum) and grown to 80% confluence. Medium was changed with serum-free medium, and after 6 h, it was replaced with fresh serum-free medium in the presence (100 ng/ml) or absence of IGFBP-3. After 24 h of incubation, cells were fixed with 2% paraformaldehyde, washed three times with PBS, permeabilized with proteinase K [10 μg/ml in 10 mM Tris-HCl (pH 7.4)] for 10 min at 37°C, and incubated with terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction mixture for 60 min at 37°C in a humidified chamber. At the end of incubation, slides were rinsed three times in PBS and were incubated with alkaline phosphatase conjugate for 30 min at 37°C. Finally, Fast Red substrate solution (2.5 mg/ml) was added to the each slide, and the slides were kept for 10 min at room temperature in the dark. After washing in PBS, sections were counterstained in hematoxylin, and apoptotic cells were counted under light microscope.

![Fig. 2](image2.png) **Identification of the M, 80,000 (80 kDa) complex as an insulin-like growth factor binding protein-3-autocrine motility factor/phosphoglucose isomerase isomerase (IGFBP-3–AMF/PGI).** In A, biotinylated IGFBP-3 was cross-linked with disuccinimidyl suberate (DSS) to proteins present in the solubilized T47D cell membrane preparation. After a pull-dowm with streptavidin-agarose, the pellets were analyzed by SDS-PAGE and transfer to nitrocellulose paper that was probed with streptavidin-horseradish peroxidase (Streptavidin-HRP, left panel) or with antibody to AMF/PGI (Anti-AMF/PGI, right panel). In B, biotinylated IGFBP-3 cross-linked with DSS to the solubilized T47D cell membrane preparation was immunoprecipitated with anti-AMF/PGI and analyzed by SDS-PAGE. After transfer, the nitrocellulose paper was probed with streptavidin-HRP. Biotinylated IGFBP-3 was included on the gel as a standard. IDc, M, in thousands.

![Fig. 3](image3.png) **Displacement curves for insulin-like growth factor binding protein-3 (IGFBP-3) and IGFBP-3:insulin-like growth factor I (IGF-I) binary complex from immobilized autocrine motility factor/phosphoglucose isomerase (AMF/PGI).** A representative displacement curve and Scatchard plot is shown. The Kd for the interaction of IGFBP-3 with AMF/PGI and binary complex with AMF/PGI was 2.59 ± 0.39 nM versus 1.25 ± 0.16 nM, respectively (P = 0.05).
Identification of AMF/PGI As an IGFBP-3 Binding Partner.

When biotinylated IGFBP-3 was incubated with T47D cell monolayers and subsequently cross-linked using DSS, three proteins with an apparent Mr of ~80,000, ~100,000, and ~125,000 were identified using streptavidin-HRP (Fig. 1A). Similar size complexes were apparent when solubilized membranes rather than intact cells were used and when MCF-7 cells rather than T47-D cells were used (data not shown). The protein with Mr ~125,000 was the least abundant. The Mr ~80,000 band was assumed to be IGFBP-3. To identify the nature of the Mr ~80,000 protein complex, solubilized membranes from T47D cells were subjected to gel permeation, ion exchange, and IGFBP-3-affinity chromatography. Fractions eluted under acidic conditions from the IGFBP-3-affinity column were analyzed by SDS-PAGE and silver staining (Fig. 1B). Ligand blotting with biotinylated IGFBP-3 identified a similar band (Fig. 1C). The Mr ~50,000 protein was identified as AMF/PGI using in-gel tryptic digestion and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF). The identity was confirmed by tandem mass spectrometry (MS/MS). Rabbit muscle PGI had an identical molecular mass (Fig. 1C). Furthermore, immunoblotting with anti-AMF antisera demonstrated the presence of AMF in the membrane fractions eluted from the IGFBP-3-affinity column (Fig. 1D).

Binding of IGFBP-3 to AMF/PGI. To confirm that the Mr ~80,000 protein complex identified by cross-linking biotinylated IGFBP-3 to the cell membranes, contained AMF/PGI, antiserum to AMF/PGI was used to immunoblot biotinylated complexes in solubilized membranes from T47D cells that had been precipitated with streptavidin-agarose (Fig. 2A). In the absence of DSS, the Mr ~80,000 band did not appear, and only biotinylated IGFBP-3 was detected. In the presence of DSS, the Mr 80,000 band was recovered by streptavidin-agarose precipitation. This band contained immunoreactive AMF/PGI (right panel, Fig. 2A). We also demonstrated that IGFBP-3 was present when the Mr ~80,000 complex was immunoprecipitated with antiserum to AMF/PGI (Fig. 2B). We used a similar strategy to demonstrate the presence of AMF/PGI in solubilized membrane preparations form MCF-7 cells (data not shown).

An immobilized ligand-based assay system was used to characterize the binding of IGFBP-3 to AMF/PGI. Fig. 3 shows the displace-

**TCA Precipitation.** Trichloroacetic acid (TCA) precipitation was used to determine the incorporation of 32P-orthophosphate into protein, both in cell lysates and conditioned medium. Cell lysate and conditioned medium were precipitated with 5% ice-cold TCA. Tubes were vortexed, and 100 μl of 1% BSA were added to the conditioned medium tubes; the tubes were vortexed again and were centrifuged for 5 min at 10,000 rpm. The precipitated pellet was washed three times with ice-cold TCA and radioactivity was counted.

**Statistical Analysis.** Data are expressed as the mean ± SE. Student's t test was used for single comparisons. For determining statistical differences between multiple groups, an ANOVA with repeated measures followed by Dunnett's t test was used.

**RESULTS**

Identification of AMF/PGI As an IGFBP-3 Binding Partner.

When biotinylated IGFBP-3 was incubated with T47D cell monolayers and subsequently cross-linked using DSS, three proteins with an apparent Mr of ~32,000, ~80,000, and ~100,000 were identified using streptavidin-HRP (Fig. 1A). Similar size complexes were apparent when solubilized membranes rather than intact cells were used and when MCF-7 cells rather than T47-D cells were used (data not shown). The protein with Mr ~100,000 was the least abundant. The Mr ~32,000 band was assumed to be IGFBP-3. To identify the nature of the Mr ~80,000 protein complex, solubilized membranes from T47D cells were subjected to gel permeation, ion exchange, and IGFBP-3-affinity chromatography. Fractions eluted under acidic conditions from the IGFBP-3-affinity column were analyzed by SDS-PAGE and silver staining (Fig. 1B). Ligand blotting with biotinylated IGFBP-3 identified a similar band (Fig. 1C). The Mr ~50,000 protein was identified as AMF/PGI using in-gel tryptic digestion and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF). The identity was confirmed by tandem mass spectrometry (MS/MS). Rabbit muscle PGI had an identical molecular mass (Fig. 1C). Furthermore, immunoblotting with anti-AMF antisera demonstrated the presence of AMF in the membrane fractions eluted from the IGFBP-3-affinity column (Fig. 1D).

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We examined the effect of pretreatment of T47D cells with AMF/PGI on the translocation and secretion of AMF/PGI. AMF/PGI is a predominantly cytosolic protein, and pretreatment with AMF/PGI is important in the translocation and secretion of AMF/PGI (data not shown).

IGFBP-3 on the phosphorylation of AMF/PGI. Pretreatment of cell monolayers reduced the amount of phosphorylated AMF/PGI associated with the cell pellet but had no effect on the total amount of immunodetectable AMF/PGI (Fig. 5A). In contrast, pretreatment with IGFBP-3 reduced both the absolute amount of AMF/PGI in the conditioned medium and the amount of 32P radiolabeling (Fig. 5B).

Under the same experimental conditions, the amount of AMF/PGI detectable on the cell monolayer using AMF/PGI antibody and radiolabeled antirabbit IgG was significantly reduced (Fig. 5C). These effects were not due to a generalized effect of IGFBP-3 on cellular metabolism because the protein content in neither the cell pellet nor conditioned medium was affected by IGFBP-3 (Table 1). Furthermore, IGFBP-3 had no effect on 32P incorporation into TCA-precipitated material (Table 1).

IGFBP-3 Inhibits AMF/PGI Binding to Cell Monolayers. AMF/PGI mediates its cytokine effects by binding to a specific cell membrane receptor. The ability of IGFBP-3 to inhibit the binding of AMF/PGI to T47D cell monolayers was investigated. Various concentrations of IGFBP-3 were added together with 50 ng/ml biotinylated AMF/PGI to T47D cell monolayers, and the amount of biotinylated AMF/PGI bound to the monolayer was determined using 125I-labeled streptavidin. At concentrations of IGFBP-3 as low as 10 ng/ml, there was significant inhibition of the binding of AMF/PGI to the cell monolayers (Fig. 6). At the highest concentration tested, 1 µg/ml IGFBP-3 reduced the binding of AMF/PGI to the cell monolayer by ∼60%. IGFBP-3:IGF-I binary complex had an effect that was similar to that of IGFBP-3 alone. Similar data were obtained when MCF-7 rather than T47D cells were used (data not shown).

IGFBP-3 Inhibits AMF/PGI-Induced Cell Migration. The ability of IGFBP-3 to inhibit AMF/PGI-induced migration of T47D cells was examined using a two-chamber technique. In the absence of added AMF/PGI, IGFBP-3 at 100 ng/ml had no significant effect on the migration of MCF-7 or T47D cells compared with the control well: 16.3 ± 3.12 versus 18.3 ± 2.9 and 15.0 ± 2.7 versus 17.4 ± 2.6 cells/filter for MCF-7 and T47D cells, respectively. AMF/PGI at a concentration of 100 pg/ml increased the migration of T47D cells by ∼4-fold (Fig. 7). This effect was completely blocked by AMF/PGI antibody that by itself had no significant effect on cell migration. IGFBP-3 also caused a dose-dependent inhibition of AMF/PGI-induced cell migration. Significant effects were demonstrable with as little as 10 ng/ml IGFBP-3, and at 100 ng/ml, IGFBP-3 reduced cell migration by ∼50%. Similar data were obtained with MCF-7 cells (data not shown). Under these conditions, IGFBP-3 at a concentration of 100 ng/ml, the absolute increase in the percentage of apoptotic cells in culture was ∼9%. In experiments in which T47D cells were used, 12.01 ± 1.80% of the cells were apoptotic versus 4.21 ± 0.56% in control wells as measured by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay. In MCF-7 cells, these numbers were 13.53 ± 1.76 versus 3.89 ± 0.63%, respectively. This represents an ∼3- to 4-fold increase in the number of apoptotic

Table 1 Effect of insulin-like growth factor binding protein 3 (IGFBP-3) on protein content and incorporation of [32P]phosphate into trichloracetic acid (TCA) precipitated material in cell lysate and conditioned medium.

<table>
<thead>
<tr>
<th></th>
<th>T47D cells</th>
<th>MCF-7 cells</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IGFBP-3</td>
</tr>
<tr>
<td>Cell lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein, µg/10^6 cells</td>
<td>5.32 ± 0.76</td>
<td>5.01 ± 0.90</td>
</tr>
<tr>
<td>TCA ppt.* cpm/µg proteins</td>
<td>89,536 ± 6,357</td>
<td>85,642 ± 5,481</td>
</tr>
<tr>
<td>Conditioned medium</td>
<td></td>
<td></td>
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<tr>
<td>Total protein, µg/ml</td>
<td>0.059 ± 0.003</td>
<td>0.057 ± 0.002</td>
</tr>
<tr>
<td>TCA ppt. cpm/µg proteins</td>
<td>1,502 ± 137</td>
<td>1,632 ± 119</td>
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* ppt., precipitable.

Fig. 6. Insulin-like growth factor binding protein-3 (IGFBP-3) inhibits binding of autocrine motility factor/phosphoglucone isomerase (AMF/PGI) to T47D cell monolayers. Cell monolayers were incubated biotinylated AMF/PGI (50 ng/ml), in the presence or absence of IGFBP-3 or the equivalent amount of IGFBP-3:insulin-like growth factor-I (IGF-I) binary complex. The amount of cell-associated AMF/PGI was determined with 125I-labeled antirabbit IgG was significantly reduced (Fig. 5A). In contrast, pretreatment with IGFBP-3 reduced both the absolute amount of AMF/PGI in the conditioned medium and the amount of 32P radiolabeling (Fig. 5B).

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2520
respectively, for the difference from AMF/PGI-treated cells. IGFBP-3 treated cells in the absence of AMF/PGI.

and P/H11011 modest at cells but the decrease in the absolute percentage of viable cells was

AMF/PGI Inhibits IGFBP-3-Induced Apoptosis. We tested the ability of AMF/PGI to inhibit IGFBP-3-induced apoptosis in T47D and MCF-7 cells. IGFBP-3 at a concentration of 100 ng/ml increased apoptosis approximately 5-fold in both MCF-7 and T47D cells (Fig. 8). In both cell lines, AMF/phosphohexose isomerase resulted in a dose-dependent inhibition of IGFBP-3-induced apoptosis.

DISCUSSION

The most abundant protein complex identified by cross-linking biotinylated IGFBP-3 to breast cancer cell membranes was identified as a Mr ~80,000 protein complex consisting of AMF/PGI bound to IGFBP-3. The formation of this complex on the cell membrane was inhibited by the presence of IGF-I. The interaction of IGFBP-3 with AMF/PGI was confirmed by coinmunoprecipitation of the protein complex from solubilized membranes. Furthermore, we demonstrated that IGFBP-3 interacted with purified AMF/PGI from rabbit muscle.

In higher species, AMF/PGI is a bifunctional molecule. In addition to its role as a glycolytic enzyme it also functions as a cytokine. As a cytokine, it stimulates motility, survival, proliferation, and maturation of a wide variety of cells (17). It has been suggested that its role as a cytokine is a prosurvival mechanism to protect glycolytically active cells from cell death-induced nutrient depletion (19). Although AMF/PGI is ubiquitously expressed, translocation to the cell membrane and secretion into conditioned medium is more commonly seen in transformed cell lines than in normal cells (17). Autocrine motility activity was initially described as a Mr ~55,000 protein present in the conditioned medium of A2058 melanoma cells and ras-transformed NIH-3T3 cells (20). Subsequent purification and microsequence data demonstrated that this activity was identical to a neurotropic factor, neuroleukin, a lymphokine produced by activated macrophages (12) that had previously been shown to be homologous to phosphoglucose isomerase (21, 22). Other biological functions of AMF/PGI include maturation of myeloid cells (23), induction of angiogenesis (24), mitogenesis and transformation of cells to a more malignant phenotype (19, 25).

Previous studies have demonstrated that the inhibitors of the catalytic activity of AMF/PGI such as mannose 6-phosphate also inhibit the cytokine function of this protein (12), suggesting that the catalytic domain overlaps the cytokine domain. We confirmed that IGFBP-3 inhibited both the catalytic activity of AMF/PGI and its ability to induce migration of breast cancer cells. IGFBP-1 and IGFBP-5, structurally similar binding proteins, did not share the ability to interact with AMF/PGI. Rather surprisingly, we demonstrate that IGF-I/IGFBP-3 had a higher binding affinity than AMF/PGI and was a more potent inhibitor of AMF/PGI than IGFBP-3 alone. Conformational changes in IGFBP-3 after binding to IGF-I have been shown to enhance the binding of IGFBP-3 to the acid-labile subunit (26), and a similar mechanism may explain the enhanced potency of the binary complex to inhibit AMF/PGI catalytic activity. However, we have previously shown that IGF-I inhibits the binding of IGFBP-3 to T47D cells (14), an observation that is consistent with reports in other cell lines (8, 9, 27). Furthermore, in the presence of IGF-I, we are unable to cross-link IGFBP-3 to AMF/PGI, and the Mr ~80,000 complex that represents IGFBP-3/AMF/PGI is not observed (14). This would sug-

Fig. 7. Insulin-like growth factor binding protein-3 (IGFBP-3) inhibits autocrine motility factor/phosphogluco isomerase (AMF/PGI)-induced migration of T47D cells. The ability of AMF/PGI, 100 pg/ml, to induced cell migration was tested in the absence (open histograms) or presence of various concentrations of IGFBP-3 (closed histograms). The data represent the mean ± SE for n = 6; * indicate P < 0.05 and P < 0.01, respectively, for the difference from AMF/PGI-treated cells.

Fig. 8. Autocrine motility factor/phosphogluco isomerase (AMF/PGI) inhibits insulin-like growth factor binding protein-3 (IGFBP-3)-induced apoptosis in MCF-7 and T47D cells. Left panel, the abundance of histone-associated DNA fragments in the presence or absence of IGFBP-3. Right panel, the effects of various concentrations of AMF/PGI on the IGFBP-3-induced increase in histone-associated DNA fragments. The data represent ± SE for n = 6; * indicate P < 0.05 and P < 0.01, respectively, for the difference from IGFBP-3 treated cells in the absence of AMF/PGI.
gest that membrane-associated AMF/PGI is unable to bind IGF-I/IGFBP-3 binary complex. AMF/PGI is devoid of a secretory signal and is secreted by the nonclassical pathway shared with some other growth factors and cytokines such as fibroblast growth factor (28) and interleukin-1β (29).

When added together, IGFBP-3 was able to inhibit both the binding of AMF/PGI to the cell monolayer and AMF/PGI-induced cell migration. However the reduction in AMF/PGI-induced cell migration was not due to IGFBP-3-induced apoptosis because IGFBP-3, when added alone under these conditions, had no significant effect on cell motility. These observations suggest that IGFBP-3 could potentially disrupt the AMF/PGI interaction with the AMF-R.

Preincubation of breast cancer cells with IGFBP-3 reduces the phosphorylation of AMF/PGI and its translocation to the membrane. Although this effect may be indirect because preincubation with IGFBP-3 may have induced changes in the metabolic state of cells and would have resulted in an increased number of apoptotic cells, there was no appreciable effect on protein content of the cells or on the incorporation of radiolabeled phosphate into cellular or secreted proteins.

Both AMF/PGI and its receptor AMF-R are overexpressed in malignant cells, and the level of expression has prognostic significance (30). For example, detection of AMF/PGI in the blood of patients with gastrointestinal, renal, and breast malignancies is associated with a worse prognosis (31), and overexpression of AMF-R has been associated with a poor prognosis in bladder carcinoma (30). IGFBP-3 has both stimulatory and proapoptotic inhibitory effects that can be IGF dependent or IGF independent. The proapoptotic, IGF-independent effects are seen in cancer cells, whereas in nontransformed cells IGF-independent proliferative effects are seen. For example, as demonstrated here and elsewhere (4, 10), IGFBP-3 stimulates apoptosis in breast cancer cells. In contrast, in MCF-10A breast epithelial cells, IGFBP-3 stimulates cell proliferation (32). This difference in the actions of IGFBP-3 may be related to the level of expression of AMF/PGI and AMF-R in malignant versus nontransformed cells. Here, we were able to demonstrate that the addition of AMF/PGI was able to overcome the proapoptotic effects of IGFBP-3 in both MCF-7 and T47D cells, suggesting that these effects of IGFBP-3 may, indeed, involve the AMF/PGI-AMF-R autocrine loop.

In summary, we have identified AMF/PGI as an abundant binding partner for IGFBP-3 on breast cancer cell membranes. The ability of IGFBP-3 to bind to, and to disrupt, the autocrine actions of AMF/PGI may be important in IGFBP-3-induced apoptosis.

REFERENCES

Insulin-Like Growth Factor Binding Protein-3 Interacts with Autocrine Motility Factor/Phosphoglucose Isomerase (AMF/PGI) and Inhibits the AMF/PGI Function

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