Interaction of plasminogen with dipeptidyl peptidase IV initiates a signal transduction mechanism which regulates expression of matrix metalloproteinase-9 by prostate cancer cells

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Both plasminogen (Pg) activation and matrix metalloproteinases (MMPs) are involved in the proteolytic degradation of extracellular matrix components, a requisite event for malignant cell metastasis. The highly invasive 1-LN human prostate tumour cell line synthesizes and secretes large amounts of Pg activators and MMPs. We demonstrate here that the Pg type 2 (Pg 2) receptor in these cells is composed primarily of the membrane glycoprotein dipeptidyl peptidase IV (DPP IV). Pg 2 has six glycoforms that differ in their sialic acid content. Only the highly sialylated Pg 2 γ , Pg 2 δ and Pg 2 ϵ glycoforms bind to DPP IV via their carbohydrate chains and induce a Ca²⁺ signalling cascade;

however, Pg 2 ϵ alone is also able to significantly stimulate expression of MMP-9. We further demonstrate that the Pg-mediated invasive activity of 1-LN cells is dependent on the availability of Pg 2 ϵ . This is the first demonstration of a direct association between the expression of MMP-9 and the Pg activation system.

Key words: calcium signalling cascade, matrix metalloproteinase-9 expression, plasminogen glycoforms, plasminogen receptors, tumour cell invasion.

INTRODUCTION

The development of an aggressive phenotype, commonly associated with the invasive behaviour of many tumours, involves the increased expression of proteinases that can digest components of the extracellular matrix (ECM), thus permitting passage of malignant cells through basement membranes and stromal barriers [1]. Among these enzymes, urinary-type plasminogen (Pg) activator (u-PA) and a variety of matrix-degrading metalloproteinases (MMPs), including MMP-2 and -9, play important roles [2–5]. Of particular relevance is the observation that these enzymes are secreted as inactive zymogens (pro-u-PA, proMMPs) which are activated extracellularly by limited proteolysis. Trace amounts of plasmin can activate pro-u-PA [4], thus generating a self-maintaining feedback mechanism in which activation of pro-u-PA catalyses the conversion of Pg into plasmin. Pg binding occurs in close proximity to the u-PA–u-PA receptor complex, and serves to facilitate Pg activation, confine plasmin to desired sites of action, and protect plasmin and its activator from their respective inhibitors [4]. Plasmin activates proMMP-2 and proMMP-9 directly either in solution [6,7] or when both MMPs are associated with the cell surface [5,8].

The regulation of the expression and activity of MMP-9 is more complex than that of most other MMPs [9]. MMP-9 is not produced constitutively by most cells [10,11], but its activity is induced by different stimuli depending on the cell type [12,13], thereby providing a means of increasing its activity in response to specific pathophysiological events. For instance, MMP-9 is expressed at high levels in human prostate cancer, but is absent from normal prostatic tissue [14,15]. Highly invasive DU-145, PC-3 and 1-LN human prostate tumour cell lines synthesize and secrete large amounts of u-PA and proMMP-9 [16,17].

In human rheumatoid synovial fibroblasts, cell binding of Pg and its activation by u-PA induces a significant rise in the cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) [18], via interaction of Pg/plasmin with the integrin α IIb β 3 and dipeptidyl peptidase IV (DPP IV) on the cell surface [19,20]. DPP IV activities are also elevated in malignant human prostate cancers [21]. DU-145 and PC-3 cells express the integrin α IIb β 3 on their surface [22]; however, expression of this integrin or of DPP IV by 1-LN cells has not been assessed. Since expression of MMP-2 by human melanoma, fibrosarcoma and ovarian cancer cells is regulated by receptor-dependent Ca²⁺ influxes [23], we investigated the possibility that a similar regulatory signal transduction mechanism participates in MMP-9 production by 1-LN cells. Pg type 2 (Pg 2) has six glycoforms that differ in their sialic acid content [24]. Extensive research has demonstrated that the sialic acid content affects not only the activation of Pg, but also its function [24–27]. In the present investigation, we studied the function of single Pg 2 glycoforms after binding to 1-LN human prostate cancer cells, and found that Pg 2 α and Pg 2 β bind to an L-lysine site-dependent receptor, whereas the highly sialylated Pg 2 γ , Pg 2 δ and Pg 2 ϵ glycoforms bind primarily to DPP IV. We also present data suggesting that DPP IV in association with Pg 2 ϵ alone regulates expression of proMMP-9.

EXPERIMENTAL

Materials

Culture media were purchased from Life Technologies Inc. (Gaithersburg, MD, U.S.A.). Fura 2 acetoxymethyl ester (fura 2/AM) was obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Two-chain, high-M_r u-PA (M_r ~ 54000) was obtained

Abbreviations used: ECM, extracellular matrix; MMP, matrix metalloproteinase; Pg, plasminogen; DPP IV, dipeptidyl peptidase IV (CD26); u-PA, urinary-type plasminogen activator; t-PA, tissue-type plasminogen activator; mAb, monoclonal antibody; FAP α , fibroblast activation protein α ; HBSS, Hanks balanced salt solution; fura 2/AM, fura 2 acetoxymethyl ester; 6-AHA, 6-aminoheptanoic acid; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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from Calbiochem (Richmond, CA, U.S.A.). The chromogenic plasmin substrate Val-Leu-Lys-*p*-nitroanilide and the chromogenic DPP IV substrate Gly-Pro-*p*-nitroanilide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other reagents used were of the highest grade available.

Antibodies

The monoclonal antibody (mAb) SZ21 (IMMUNOTECH, Inc., Westbrook, ME, U.S.A.) binds specifically to the platelet GPIIIa (β 3)-subunit [28]. Anti-DPP IV mAb clone 236.3 [29] was generously donated by Dr Douglas C. Hixson (Brown University, Providence, RI, U.S.A.). Anti-u-PA mAb 390 and goat anti-[human recombinant tissue-type Pg activator (t-PA)] IgG (both anti-catalytic) were purchased from American Diagnostica (Greenwich, CT, U.S.A.). Anti-[fibroblast activation protein α (FAP α)] mAb F19 [30] was a gift from Dr Pilar Garin-Chesa (Thomae G.m.b.H., Biberach, Germany). The anti-catalytic anti-MMP-9 mAb, clone 6-6B [31], was purchased from Oncogene Research Products (Cambridge, MA, U.S.A.). Goat anti-(mouse IgG)-alkaline phosphatase conjugate antibodies were purchased from Sigma Chemical Co.

Proteins

Pg was purified from human plasma by affinity chromatography on L-lysine-Sepharose [32] and separated into its two classes of isoforms, Pg 1 and Pg 2, by affinity chromatography on concanavalin A-Sepharose [33]. Fractionation of Pg 2 into its six glycoforms and measurement of sialic acid content were performed as described previously [24]. The mean distribution of the first five Pg 2 glycoforms in native Pg 2 was calculated from the yields obtained for each purified glycoform using chromatofocusing on a Mono P column linked to an FPLC system [24] from five separate preparations. The proportion of Pg 2 ϕ was calculated from the amount of protein obtained after chromatography of native Pg 2 on a *Sambucus nigra* agglutinin lectin-Sepharose column [34], and also represents the mean value of five separate preparations. Radioiodination was carried out by the method of Markwell [35]. Radioactivity was measured in a Pharmacia LKB Biotechnology 1272 γ -radiation counter (Pharmacia, Rockville, MD, U.S.A.). Incorporation of ^{125}I was at a level of $\sim 8 \times 10^6$ c.p.m./nmol of protein. ^{125}I -labelled Pg was re-purified by affinity chromatography on L-lysine-Sepharose and then used for the binding experiments.

Cell cultures

The human prostate tumour cell line 1-LN was grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G and 100 ng/ml streptomycin.

Purification of DPP IV from 1-LN cell membranes

Cells grown in 20 culture flasks (150 cm²) were detached with 10 mM EDTA in Hanks balanced salt solution (HBSS) and pelleted by centrifugation. The cell pellet was suspended in 10 ml of 20 mM Hepes, pH 7.2, containing 0.25 M sucrose and the following proteinase inhibitors (each at 0.5 mg/ml): antipain-HCl, bestatin, chymostatin, *trans*-epoxysuccinyl-L-leucylamid-(4-guanidino)butane (E-64), leupeptin, pepstatin, *o*-phenanthroline and aprotinin. Cells were lysed by sonication on ice (five 10 s bursts with 30 s intervals). All procedures were performed at 4 °C. The homogenate was centrifuged at 800 *g* for 15 min to remove unbroken cells and nuclei, followed by centrifugation of

the supernatant at 50000 *g* for 1 h. The pellet containing cell membranes was resuspended in 20 mM Tris/HCl, pH 8.0, containing 1% (v/v) Triton X-100 to solubilize membranes and centrifuged again at 50000 *g* for 30 min to remove insoluble materials. DPP IV activity in this supernatant and in all the subsequent purification steps was monitored by a chromogenic assay using the DPP IV substrate Gly-Pro-*p*-nitroanilide [36]. The enzyme was sequentially purified to homogeneity using DEAE-Sepharose ion-exchange chromatography and Gly-Leu-Sepharose affinity chromatography [37], followed by chromatography on concanavalin A-Sepharose and gel filtration on a Sepharose S-200 column. These steps yielded fully active DPP IV ($\sim 40 \mu\text{g}/10^9$ cells). Electrophoretic analysis showed an essentially homogeneous protein. A sample of the protein was analysed by matrix-assisted laser desorption ionization MS, and the mass spectrometric peptide maps obtained (30 peptides) were used to identify DPP IV in the OWL Protein database release 29.6 [38,39].

Protein sequence analysis

The proteins (100 pmol) were sequenced by automatic Edman degradation in a gas/liquid phase sequencer (model 477A; Applied Biosystems, Inc., Foster City, CA, U.S.A.) with online phenylthiohydantoin analysis using HPLC (model 120A; Applied Biosystems). The instruments were operated as recommended in the user bulletins and manuals distributed by the manufacturer.

Ligand binding analysis

Cells were grown in tissue culture plates until the monolayers were confluent. Prior to use in binding assays, the cells were washed in HBSS. All binding assays were performed at 4 °C in RPMI 1640 containing 2% (w/v) BSA. Increasing concentrations of ^{125}I -labelled Pg 2 glycoforms were incubated with cells for 60 min in 48-well or 96-well culture plates. Free ligand was separated from bound ligand by aspirating the incubation mixture and washing the cell monolayers rapidly three times with RPMI 1640 containing 2% BSA. The cells were then lysed with 0.1 M NaOH, and bound radioactivity was determined in a Pharmacia LKB Biotechnology 1272-gamma counter. Molecules of ligand bound were calculated after subtraction of non-specific binding measured in the presence of 100 μM unlabelled Pg 2. Estimates of dissociation constant (K_d) values and maximal binding of Pg 2 glycoforms (B_{max}) were determined by fitting data directly to the Langmuir isotherm using the statistical program SYStat[®] for Windows.

Solid-phase radioligand binding studies

To study specific binding of Pg 2 glycoforms to immobilized DPP IV purified from 1-LN cells, 96-well strip plates were coated with DPP IV (1 $\mu\text{g}/\text{ml}$ in 0.1 M sodium carbonate, pH 9.6; 200 $\mu\text{l}/\text{well}$; 37 °C; 2 h). After coating, plates were washed with 200 μl of 10 mM sodium phosphate/100 mM NaCl, pH 7.4, containing 0.05% Tween-80 (PBS-Tween) to remove unbound protein. Non-specific sites were blocked by incubation with PBS-Tween containing 2% (w/v) BSA at room temperature for 1 h. Plates were rinsed twice with 200 μl of PBS-Tween, air dried, and stored at 4 °C. For assays, increasing concentrations of ^{125}I -labelled Pg 2 glycoforms, with or without a 50-fold excess of unlabelled ligand, were added to triplicate wells and incubated at 37 °C for 1 h. Following incubation, the supernatants were removed and the plates were rinsed three times with 200 μl of

PBS-Tween. Wells were stripped from the plates and radioactivity was measured. Specific binding was calculated by subtraction of non-specific binding measured in the presence of unlabelled ligand.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by digital imaging microscopy using the fluorescent indicator fura 2/AM as previously described [18].

Gelatin zymography

Protein samples were electrophoresed on gelatin-containing 0.75 mm thick 10% (w/v) polyacrylamide gels in the presence of SDS under non-reducing conditions [40]. After completion of the electrophoretic run, the gels were incubated with two changes of 2.5% (v/v) Triton X-100 for 1 h, followed by incubation for 18 h at 37 °C in 0.1 M glycine/NaOH, pH 8.3, containing 1 mM $CaCl_2$ and 0.1 M $ZnCl_2$, before staining with Coomassie Brilliant Blue R-250 to visualize the lysis bands.

MMP-9 activity in solution

MMP-9 activity was measured in tissue culture supernatants by quantitative zymography [41] using as a standard MMP-9 purified by affinity chromatography on gelatin-Sepharose from 1-LN cell conditioned medium (10 litres) [42]. Conditioned medium (50 μ l) from 1-LN cell monolayers in 48-well culture plates (1.7×10^6 cells/well), incubated with Pg 2 glycoforms and/or inhibitors of Pg binding or activation, were electrophoresed on gelatin-containing gels, and the degree of lysis was quantified using a Gelman ACD-15 Automatic Computing Densitometer (Gelman Instrument Company, Ann Arbor, MI, U.S.A.). Values were determined by integrating the density of the selected bands, and expressed in units $\cdot$ mm². Each gel was scanned three times, and the average value of the integrated density of the bands was used to determine levels of MMP-9 from calibration curves constructed with purified active MMP-9 electrophoresed under the same conditions. The statistical analysis of the data was performed on an IBM 433 DX/S computer using the program SYSTAT® for Windows 95. The statistical significance of differences between means was evaluated by Student's *t*-test.

MMP-9 was positively identified in conditioned medium by electrophoretic separation in 10% (w/v) polyacrylamide gels (SDS/PAGE), electroblotting of the electrophoresed proteins to nitrocellulose membranes, and reaction with an anti-MMP-9 mAb (1 μ g/ml) followed by reaction with a secondary alkaline phosphatase-conjugated anti-mouse IgG). Detection was performed by reaction with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of Nitro Blue Tetrazolium (1 mM each) in 10 mM Tris/HCl, pH 8.5.

Gel electrophoresis

Electrophoresis was performed on polyacrylamide gels (1.2 mm thick; 14 cm $\times$ 10 cm) containing 0.1% SDS. A discontinuous Laemmli buffer system was used [43]. Visualization of the proteins was carried out by staining the gel with 0.25% Coomassie Brilliant Blue R-250 in 45% (v/v) methanol/10% (v/v) acetic acid. Transfer to nitrocellulose paper was carried out by the Western blot method [44]. The dye-conjugated M_r markers (Bio-Rad, Richmond, CA, U.S.A.) used were: myosin (M_r 218000), β -galactosidase (M_r 134000), BSA (M_r 84000), carbonic anhydrase (M_r 44000) and soybean trypsin inhibitor (M_r 32000).

Flow cytometry

1-LN cells were grown at 37 °C in RPMI 1640 containing 10% (v/v) fetal bovine serum as adherent monolayers. Cells were detached by incubation for 5 min at 37 °C with Ca^{2+} - and Mg^{2+} -free PBS containing 10 mM EDTA, and then pelleted. Cells were resuspended in ice-cold staining buffer (Phenol Red-free HBSS, 1% BSA and 0.1% NaN_3) at a concentration of 1×10^7 cells/ml. Aliquots (100 μ l) of these cell suspensions were incubated for 90 min on ice with an appropriate dilution of FITC-conjugated anti-(human DPP IV), FITC-conjugated anti-[human GPIIIa (β 3)] or a FITC-conjugated isotype control murine mAb. For analyses of cell-surface FAP α , cells were first incubated on ice with the anti-FAP α mAb F19 for 90 min, and then for an additional 90 min with a FITC-conjugated anti-(mouse IgG). Cells were then rinsed three times with ice-cold staining buffer, resuspended in ice-cold 10% formalin, and stored in the dark at 4 °C until analysis by flow cytometry. The mean relative fluorescence after excitation at a wavelength of 408 nm was determined for each sample on a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) and analysed with CELLQUEST™ software (Becton-Dickinson).

RNA isolation

To determine changes in MMP-9 mRNA induced by Pg, 1-LN cells were grown in 48-well culture plates (1.7×10^6 cells/well) and incubated with each individual Pg 2 glycoform for 24 h at 37 °C. Cell monolayers were then rinsed twice in serum-free RPMI 1640, and total RNA was extracted by a single-step method using an RNeasy Mini kit (Qiagen, Chatsworth, CA, U.S.A.), according to the manufacturer's instructions.

Measurement of MMP-9 mRNA levels by reverse transcription-PCR

Total RNA was reverse-transcribed with 1 μ g of RNA in a 20 μ l reaction mixture, using M-MLV (Moloney murine leukaemia virus) reverse transcriptase (200 units) and oligo(dT) as primer for 1 h at 42 °C. The resulting cDNA (5 μ l) was used as a template and a 212 bp segment of the MMP-9 cDNA was amplified, using a 24-mer upstream primer (5'-AGTTGAACC-AGGTGGACCAAGTGG-3') identical to positions 2079–2102, and a 29-mer downstream primer (5'-AACAAAAACAAAG-GTGAGAAGAGAGGGC-3') complementary to positions 2270–2298, of the human MMP-9 mRNA [45]. A 600 bp segment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH; constitutive internal control) cDNA was co-amplified, using a 24-mer upstream primer (5'-CCACCCATGGCAAATTCC-ATGGCA-2') identical to positions 212–235, and a 24-mer downstream primer (5'-TCTAGACGGCAGGTCAGGTCC-ACC-3'), complementary to positions 786–809, of the human GAPDH mRNA [46]. Amplification was carried out in a Techne Thermal Cycler PHC-3 for 28 cycles (one cycle: 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s). PCR products were analysed on a 1.2% (w/v) agarose/ethidium bromide gel. The gels were photographed and the intensity of the individual MMP-9 and GAPDH mRNA bands was measured by laser densitometric scanning, using a Molecular Dynamics Personal Densitometer. Changes in MMP-9 mRNA levels were expressed as a relative ratio of MMP-9 mRNA/GAPDH mRNA band intensities.

In vitro invasion assay

Invasive activity *in vitro* was assessed by determining the ability of 1-LN cells to invade Matrigel® [47]. Polycarbonate filters (8 μ m pore size; Becton Dickinson) were coated with Matrigel

(12 µg/filter) and placed in a modified Boyden chamber. Cells (1×10^5) were added to the upper chamber in serum-free RPMI 1640 medium, or medium containing purified Pg 2 glycoforms in the absence or presence of anti-DPP IV, anti-u-PA or anti-MMP-9 IgGs, and incubated for 48 h in a humidified atmosphere. Following incubation, non-invading cells were removed from the upper chamber with a cotton swab, and filters were excised and stained with Cyto-Quik[®] (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Cells on the lower surface of the filter were enumerated using an ocular micrometer; a minimum of five high-powered fields were counted. Each experiment was performed twice with triplicate samples.

RESULTS

Binding of single Pg 2 glycoforms to 1-LN human prostate tumour cells

Binding of ¹²⁵I-labelled single Pg 2 glycoforms to 1-LN cells was determined as described in the Experimental section. Native Pg 2 has six glycoforms, which differ in their sialic acid content [24]. Binding experiments (Figure 1) showed that Pg 2α, Pg 2β, Pg 2γ, Pg 2δ and Pg 2ε (containing 1.3, 2.2, 2.95, 5.77 and 5.34 mol of

Figure 1 Binding of individual Pg 2 glycoforms to 1-LN human prostate tumour cells

Increasing concentrations of ¹²⁵I-labelled Pg 2α (○), Pg 2β (●), Pg 2γ (Δ), Pg 2δ (▲), Pg 2ε (□) or Pg 2φ (■) were added to 1-LN cells. Molecules of ligand bound were calculated after subtraction of non-specific binding measured in the presence of a 50-fold excess of non-labelled ligand, as described in the Experimental section. Data represent means ± S.D. from experiments performed in triplicate.

Table 1 Binding of Pg 2 glycoforms to 1-LN cells

1-LN cell monolayers in 96-well strip culture plates (2.0×10^4 cells/well) were incubated with serum-free RPMI 1640 in the presence of increasing concentrations of ¹²⁵I-labelled Pg 2 glycoforms. The assays and calculations of the distribution of Pg 2 glycoforms and binding parameters were performed as described in the Experimental section. Data shown represent means ± S.D. from experiments performed in triplicate.

Glycoform	Distribution (%)	<i>K_d</i> (nM)	10 ⁻⁵ × Binding (molecules/cell)
Pg 2α	16.60 ± 2.35	24.3 ± 1.33	13.0 ± 4.6
Pg 2β	13.80 ± 1.63	7.4 ± 1.86	8.3 ± 1.3
Pg 2γ	22.10 ± 2.17	10.6 ± 1.43	8.6 ± 1.7
Pg 2δ	30.92 ± 3.41	13.6 ± 1.04	18.1 ± 2.4
Pg 2ε	13.08 ± 1.16	3.8 ± 0.83	9.4 ± 0.8
Pg 2φ	3.50 ± 0.85	No binding

Figure 2 Inhibition of binding of individual Pg 2 glycoforms to 1-LN cells

(A) Cells were incubated in serum-free RPMI 1640 with a single concentration (0.1 µM) of ¹²⁵I-labelled Pg 2α (○), Pg 2β (●), Pg 2γ (Δ), Pg 2δ (▲) or Pg 2ε (□) in the presence of increasing concentrations of 6-AHA. (B) Cells were incubated in serum-free RPMI 1640 with a single concentration (0.1 µM) or ¹²⁵I-labelled Pg 2α (○), Pg 2β (●), Pg 2γ (Δ), Pg 2δ (▲) or Pg 2ε (□) in the presence of increasing concentrations of L-lactose. Data represent means ± S.D. from experiments performed in triplicate.

sialic acid/mol of Pg respectively) bind to 1-LN cells in a dose-dependent manner, with high affinity and to a large number of sites (Table 1). Pg 2φ (13.65 mol of sialic acid/mol of Pg) does not bind to 1-LN cells.

In order to assess the mechanism of binding of the individual Pg 2 glycoforms to 1-LN cells, we studied activation by cells incubated with each glycoform in the presence of 6-amino-hexanoic acid (6-AHA) and L-lactose. The anti-fibrinolytic amino acid 6-AHA prevents the interaction of Pg L-lysine binding sites with several cell-membrane-associated components [48,49]. L-Lactose is a sugar which intereferes with the binding of Neu5Ac (α2-3) or (α2-6) residues to sialic acid binding proteins [50] and inhibits binding of native Pg 2 to DPP IV on the surface of rheumatoid synovial fibroblasts [20]. Incubation of the cells with single Pg 2 glycoforms in the presence of increasing concentrations of 6-AHA inhibited the binding of Pg 2α and Pg 2β (Figure 2A), whereas increasing concentrations of L-lactose inhibited the binding of Pg 2γ, Pg 2δ and Pg 2ε (Figure 2B). Taken together, these experiments suggest that Pg 2α and Pg 2β bind to 1-LN cells via their L-lysine binding sites, and that Pg 2γ, Pg 2δ and Pg 2ε bind via their carbohydrate chains. The activation of Pg 2 glycoforms is inhibited by anti-u-PA antibodies, but is not affected by anti-t-PA antibodies, suggesting that u-PA is the

Figure 3 FACS analyses of 1-LN cells

(A) Cells were incubated with an FITC-conjugated anti-(human DPP IV) murine mAb (solid line) or an FITC-conjugated isotype control murine mAb (broken line). (B) Cells were incubated with an FITC-conjugated anti-[human GPIIIa ($\beta 3$)] murine mAb (solid line) or an FITC-conjugated isotype control murine mAb (broken line). (C) Cells were incubated with an anti-(human FAP α) mAb, F19, followed by an FITC-conjugated anti-(mouse IgG) (solid line) or an FITC-conjugated isotype control murine mAb (broken line).

primary Pg activator at the surface of 1-LN cells (results not shown).

Analyses of binding of antibodies against DPP IV, GPIIIa ($\beta 3$) and FAP α to the surface of 1-LN cells by flow cytometry

1-LN cells were analysed by FACS, as described in the Experimental section. The mAbs SZ21, specific for the platelet GPIIIa ($\beta 3$) antigen, and clone 236.3, specific for human DPP IV, were used for these experiments. As determined by FACS of 1-LN cells treated with FITC-labelled IgGs, cells reacted with the anti-DPP IV antibody (Figure 3A), whereas the cells showed no detectable GPIIIa ($\beta 3$) antigen on their surface (Figure 3B). In rheumatoid synovial fibroblasts, the integrin $\beta 3$ serves as a L-lysine binding site receptor for Pg, whereas DPP IV is a Pg sialic acid receptor [19,20]. The absence of $\beta 3$ from 1-LN cells suggests a different L-lysine binding site for Pg in these cells.

DPP IV shares 48% amino acid sequence identity with human FAP α [51], a cell surface antigen which is selectively expressed in reactive stromal fibroblasts of epithelial cancers, and malignant bone and soft tissue sarcoma cells [52]. Since DPP IV and FAP α

Figure 4 Binding of individual Pg 2 glycoforms to immobilized DPP IV isolated from 1-LN cell membranes

(A) DPP IV ($1 \mu\text{g/ml}$) from 1-LN cell membranes was used to coat 96-well plates. Increasing concentrations of ^{125}I -labelled Pg 2 α ($\circ$), Pg 2 β ($\bullet$), Pg 2 γ (Δ), Pg 2 δ ($\blacktriangle$), Pg 2 ϵ ($\square$) or Pg 2 ϕ ($\blacksquare$) were added to triplicate wells and incubated at 22°C for 1 h. Bound Pg was quantified as described in the Experimental section. Data represent means $\pm$ S.D. of experiments performed in triplicate. Inset: SDS/10% PAGE of purified DPP IV ($5 \mu\text{g}$) under reducing conditions. Lane 1, Coomassie Brilliant Blue R-250-stained gel; lane 2, blot incubated with anti-DPP IV IgG (mAb 236.3) followed by reaction with an alkaline phosphatase-conjugated secondary IgG. (B) Inhibition of binding of ^{125}I -labelled Pg 2 γ (Δ), Pg 2 δ ($\blacktriangle$) or Pg 2 ϵ ($\square$) ($0.1 \mu\text{M}$) to immobilized DPP IV by increasing concentrations of L-lactose. Bound Pg was quantified as described in the Experimental section.

share the amino acid sequence LQWLRR [51], which in rheumatoid synovial fibroblast DPP IV serves as the binding site for Pg carbohydrate chains [20], we investigated the expression of FAP α on the surface of 1-LN cells. We used mAb F19, which is specific for FAP α but non-cross-reactive with DPP IV [52,53]. 1-LN cells treated with mAb F19 and then analysed by FACS showed no detectable FAP α on their surface (Figure 3C).

Binding of Pg 2 glycoforms to immobilized DPP IV isolated from 1-LN cell membranes

Once identified as a Pg receptor, DPP IV from 1-LN cell membranes was purified to homogeneity as described in the Experimental section. Electrophoretic analysis of the protein is shown in Figure 4(A). A Coomassie Brilliant Blue R-250 stain of the electrophoresed material (Figure 4A, inset, lane 1) shows a major protein band in the $M_r \sim 120000$ size range. A blot

Figure 5 $[Ca^{2+}]_i$ responses of 1-LN cells to the binding of individual Pg 2 glycoforms

Cells were preloaded with $4 \mu\text{M}$ fura 2/AM for 20 min at 37°C , and changes in $[Ca^{2+}]_i$ were measured as described in the Experimental section. Arrows indicate the times of addition of each individual Pg 2 glycoform ($0.1 \mu\text{M}$). (A)–(F) Effects of Pg glycoforms alone: (A) 2α , (B) 2β , (C) 2γ , (D) 2δ , (E) 2ϵ , (F) 2ϕ . (G)–(I) Effects of PG glycoforms in the presence of L-lactose (100 mM): (G) 2γ , (H) 2δ , (I) 2ϵ .

binding assay with mAb clone 236.3, specific for DPP IV [26], showed reactivity only with the $M_r \sim 120000$ protein band (Figure 4A, inset, lane 2). DPP IV immobilization on cell culture plates and binding assays of Pg 2 glycoforms were performed as described in the Experimental section. Only Pg 2γ , Pg 2δ and Pg 2ϵ bound to this DPP IV in a dose-dependent and saturable manner (Figure 4A). No specific binding was observed with Pg 2α , Pg 2β or Pg 2ϕ . Binding of each individual ^{125}I -labelled Pg 2γ , Pg 2δ and Pg 2ϵ ($0.1 \mu\text{M}$ each) to DPP IV in the presence of increasing concentrations of L-lactose was progressively inhibited (Figure 4B), suggesting that Pg sialic acid residues are involved in this interaction. Since Pg 2γ , Pg 2δ and Pg 2ϵ represent over 65% of the distribution of Pg 2 glycoforms, and since Pg 2ϕ is unable to bind (Table 1), these results suggest that DPP IV is the primary Pg 2 receptor in 1-LN cells.

$[Ca^{2+}]_i$ response to Pg 2 glycoform binding on the surface of 1-LN cells

We measured changes in $[Ca^{2+}]_i$ after binding of each individual Pg 2 glycoform to the surface of 1-LN cells. Pg 2α and Pg 2β did not produce any changes (Figures 5A and 5B respectively). However, binding of Pg 2γ , Pg 2δ or Pg 2ϵ elicited a $[Ca^{2+}]_i$ response (Figures 5C, 5D and 5E respectively). No response was observed with Pg 2ϕ (Figure 5F). Similarly, cells were incubated at 37°C for 1 h with anti-u-PA or anti- $\beta 3$ IgGs ($100 \mu\text{g}/\text{ml}$), which inhibit enzymic activity, prior to the addition of the highly sialylated glycoforms (Pg 2γ , Pg 2δ and Pg 2ϵ). Neither cell population demonstrated major changes in their $[Ca^{2+}]_i$ responses (results not shown). However, L-lactose (100 mM), which prevents the interaction of Pg carbohydrate chains with DPP IV [24], was able to inhibit the response induced by Pg 2γ , Pg 2δ or Pg 2ϵ (Figures 5G, 5H and 5I respectively). A similar inhibition

of the $[Ca^{2+}]_i$ response (results not shown) was observed when the cells were preincubated with the anti-DPP IV mAb 236.3 ($50 \mu\text{g}/\text{ml}$) before addition of these glycoforms. These results are consistent with the observations reported above, suggesting that the $[Ca^{2+}]_i$ response is the result of a direct interaction between the highly sialylated glycoforms (Pg 2γ , Pg 2δ and Pg 2ϵ) and DPP IV on the cell surface, and does not require Pg activation.

Effect of Pg on the expression of MMP-9 by 1-LN cells

Cells were seeded into 48-well culture plates and grown in RPMI 1640 medium containing 10% (v/v) fetal bovine serum. Confluent monolayers were then incubated for 24 h with quiescent culture medium containing RPMI 1640 and 0.5% (v/v) fetal bovine serum. Each individual Pg 2 glycoform ($0.1 \mu\text{M}$) was added in triplicate to cell monolayers in $300 \mu\text{l}$ of serum-free RPMI 1640 and incubated for 24 h at 37°C . Culture medium was collected to measure secretion of MMP-9, as described in the Experimental section. Prior to analyses of the MMP-9 secreted into the medium by 1-LN cells in the presence of individual Pg 2 glycoforms, we purified MMP-9 from conditioned medium (5 litres) by the technique of Masure et al. [42]. Analyses of the purified MMP-9 are shown in Figure 6. An electrophoretic analysis of the purified protein showed a major band of $M_r \sim 85000$ and a minor band of $M_r \sim 95000$ (Figure 6, lane 1). An electroblot analysis with an anti-MMP-9 mAb showed reaction of the antibody with both the $M_r \sim 85000$ and 95000 proteins (Figure 6, lane 2). Gelatin zymography of the proteins showed activity only in association with the $M_r \sim 85000$ protein (Figure 6, lane 3). N-terminal sequence analysis revealed the sequence FQTFEGDL [42], corresponding to the N-terminal sequence of active MMP-9. A similar analysis of the $M_r \sim 95000$ protein yielded the sequence APRQRQ, corresponding to the N-terminal

Figure 6 Analysis of MMP-9 purified from 1-LN cell conditioned medium

Protein samples (5 μ g) were resolved in a continuous SDS/10%-polyacrylamide gel and electroblotted on to a nitrocellulose membrane, as described in the Experimental section. Lane 1, Coomassie Brilliant Blue R-250-stained gel; lane 2, electroblot incubated with an anti-MMP-9 mAb; lane 3, gelatinolytic activity of the proteins. The N-terminal sequences of the major protein bands are shown at the left side of lane 1.

sequence of proMMP-9. These results suggest that most of the MMP-9 secreted into the culture medium by 1-LN cells is in the active form.

We then proceeded to analyse the secretion of MMP-9 into the medium by 1-LN cells incubated with each individual Pg 2 glycoform in serum-free culture medium, using the purified MMP-9 as a standard for quantification by gelatin zymography. These analyses (Figure 7A) show a major band of active protein of $M_r \sim 85000$. Quantification of this protein (Table 2) demonstrated a 3-fold stimulation of active MMP-9 secretion by 1-LN

Figure 8 Effects of anti-DPP IV IgG on the expression of MMP-9 induced by highly sialylated Pg 2 glycoforms

Cell monolayers in 48-well culture plates (1.7×10^6 cells/well) were incubated in serum-free RPMI 1640 with each individual Pg 2 glycoform (0.1 μ M) in the absence or presence of anti-DPP IV IgG (50 μ g/ml) in a volume of 0.3 ml at 37 $^{\circ}$ C for 24 h. (A) Zymographic analysis of conditioned medium (50 μ l) from cells incubated with Pg 2 γ , Pg 2 δ or Pg 2 ϵ in the absence (lanes 1, 2 and 3 respectively) or presence (lanes 4, 5 and 6 respectively) of anti-DPP IV IgG. (B) Western blot analysis of conditioned medium from cells incubated with anti-DPP IV IgG and highly sialylated Pg 2 γ , Pg 2 δ or Pg 2 ϵ . The blots were treated with anti-MMP-9 IgG. Individual Pg 2 glycoforms are identified below the lanes.

Figure 7 Effects of Pg 2 glycoforms on the expression of MMP-9 by 1-LN cells

Cell monolayers in 48-well culture plates (1×10^6 cells/well) were incubated with serum-free RPMI 1640 in the absence or presence of purified Pg 2 glycoforms (0.1 μ M) in a volume of 0.3 ml at 37 $^{\circ}$ C for 24 h. Both zymography and identification of MMP-9 by Western blot analyses in conditioned medium were performed as described in the Experimental section. Zymographic analysis was carried out of conditioned medium (50 μ l) of cells incubated with each individual Pg 2 glycoform in the absence (A) or presence (C) of L-lactose (100 mM). Western blot analysis was carried out of conditioned medium (50 μ l) of cells incubated with each individual Pg 2 glycoform in the absence (B) or presence (D) of L-lactose (100 mM). Individual Pg 2 glycoforms are identified below the lanes.

Table 2 MMP-9 activity in serum-free culture medium of 1-LN cells incubated with Pg 2 glycoforms

1-LN cell monolayers in 48-well culture plates (1.7×10^6 cells/well) were incubated with serum-free RPMI 1640 in the absence or presence of purified Pg 2 glycoforms (0.1 μ M) in a volume of 0.3 ml at 37 $^{\circ}$ C for 24 h. The effects of single Pg 2 glycoforms in the presence of 6-AHA (100 mM) or L-lactose (100 mM) were also assessed. Anti-DPP IV IgG was used at a final concentration of 50 μ g/ml. The medium was collected and aliquots (50 μ l) were assayed for active MMP-9. Values represent means $\pm$ S.D. of three separate experiments. The statistical significance of differences between means was evaluated by Student's *t* test. nd, not determined.

Addition	Active MMP-9 (ng/ml)						
	None	Pg 2 α	Pg 2 β	Pg 2 γ	Pg 2 δ	Pg 2 ϵ	Pg 2 ϕ
None	0.58 $\pm$ 0.12	0.59 $\pm$ 0.16	0.59 $\pm$ 0.13	0.60 $\pm$ 0.17	0.62 $\pm$ 0.18	1.80 $\pm$ 0.21	0.63 $\pm$ 0.14
6-AHA	0.61 $\pm$ 0.14	0.59 $\pm$ 0.17	0.55 $\pm$ 0.13	0.63 $\pm$ 0.21	0.58 $\pm$ 0.14	1.82 $\pm$ 0.23	0.57 $\pm$ 0.11
L-Lactose	0.15 $\pm$ 0.03	0.11 $\pm$ 0.02	0.17 $\pm$ 0.04	0.16 $\pm$ 0.03	0.19 $\pm$ 0.04	0.15 $\pm$ 0.05	0.11 $\pm$ 0.02
Anti-DPP IV mAb	nd	nd	nd	0.58 $\pm$ 0.12	0.56 $\pm$ 0.11	0.49 $\pm$ 0.14	nd

Figure 9 Pg-induced MMP-9 mRNA expression in cultured 1-LN cells

1-LN cell monolayers in 48-well culture plates (1.7×10^6 cells/well) were incubated in serum-free RPMI 1640 with each individual Pg 2 glycoform ($0.1 \mu\text{M}$) in a volume of 0.3 ml at 37°C for 24 h. Isolation of total cytoplasmic RNA and measurements of MMP-9 mRNA by reverse transcription-PCR were performed as described in the Experimental section. Ethidium bromide-stained gels were photographed and analysed by laser densitometric scanning. MMP-9 mRNA levels were expressed as relative MMP-9 mRNA/GAPDH mRNA ratios. Values represent means $\pm$ S.D. of three separate experiments, each carried out in duplicate.

cells incubated with Pg 2 ϵ when compared with cells incubated with other Pg 2 glycoforms or with culture medium ($P < 0.001$). Samples of these conditioned media were also subjected to SDS/PAGE under reducing conditions, electroblotted on to nitrocellulose membranes and then treated with an anti-MMP-9 mAb (Figure 7B). These studies also suggested that only Pg 2 ϵ stimulated the production of MMP-9. Cells co-incubated with 6-AHA (100 mM) and individual Pg 2 glycoforms did not show any major changes in the production of MMP-9 when compared with controls (Table 2). A zymogram of conditioned media from cells incubated with each individual Pg 2 glycoform in the presence of L-lactose (100 mM) (Figure 7C) shows an average decrease in the production of MMP-9 for every Pg 2 glycoform, with the exception of Pg 2 ϵ , which caused a 12-fold decrease in the production of MMP-9 ($P < 0.0001$) (Table 2), to levels almost undetectable in an electroblot treated with an anti-MMP-9 mAb (Figure 7D). A 4-fold decrease in the production of MMP-9 by cells co-incubated with anti-DPP IV mAb 236.3 and Pg 2 ϵ ($P < 0.001$) (Table 2) was clearly observed in the conditioned medium (lane 6 in Figures 8A and 8B respectively).

Measurements of the relative changes in MMP-9 mRNA levels (Figure 9) showed a significant increase in the expression of MMP-9 mRNA in cells incubated with Pg 2 ϵ . Cells incubated with Pg 2 α , Pg 2 β , Pg 2 γ or Pg 2 δ glycoforms, however, did not show a significant change in their relative mRNA levels (MMP-9 mRNA/GAPDH mRNA) when compared with control cells incubated with serum-free medium alone. Cells co-incubated with Pg 2 ϵ and a binding inhibitory anti-DPP IV IgG did not show a change in the relative MMP-9 mRNA levels when compared with control cells incubated with serum-free medium alone. Taken together, these results suggest that Pg 2 ϵ not only significantly stimulates expression of MMP-9, but is also involved in its activation.

Effect of Pg on 1-LN cellular invasion

Pg enhances the ability of the prostate cancer PC-3 and DU-145 cell lines to penetrate the synthetic basement membrane Matrigel® [54]. To determine whether Pg regulates invasion via secretion of MMP-9, 1-LN cells were incubated with different inhibitory antibodies in the presence of purified Pg 2 glycoforms.

Table 3 Effects of Pg 2 glycoforms on the invasiveness of 1-LN cells *in vitro*

1-LN cells (at a cell density of 1×10^5) were added to a modified Boyden chamber containing an $8 \mu\text{m}$ pore filter coated with Matrigel ($12 \mu\text{g}/\text{filter}$) in the absence or presence of purified Pg 2 glycoforms ($0.1 \mu\text{M}$), as described in the Experimental section. Anti-DPP IV, anti-u-PA and anti-MMP-9 IgGs were used at final concentrations of 50, 100 and $20 \mu\text{g}/\text{ml}$ respectively. After incubation at 37°C for 24 h, filters were excised, non-invading cells were removed from the top surface of the membrane, staining was carried out with Cyto-Quik™, and invading cells were enumerated by using an ocular micrometer and counting a minimum of five high-powered fields. Data shown represent means $\pm$ S.D. from experiments performed in triplicate. nd, not determined.

Ligand	Relative invasion (number of cells/field)			
	Serum-free medium	+ Anti-DPP IV IgG	+ Anti-u-PA IgG	+ Anti-MMP-9 IgG
None	8.3 ± 2.6	9.4 ± 3.2	5.8 ± 1.5	2.6 ± 1.3
Pg 2 α	13.5 ± 3.4	nd	nd	nd
Pg 2 β	9.4 ± 2.1	nd	nd	nd
Pg 2 γ	7.8 ± 1.6	4.6 ± 2.7	5.3 ± 2.8	2.1 ± 1.2
Pg 2 δ	9.2 ± 2.5	10.4 ± 3.1	7.8 ± 2.6	1.8 ± 1.0
Pg 2 ϵ	46.6 ± 5.4	3.1 ± 1.6	2.1 ± 1.3	1.5 ± 1.0
Pg 2 ϕ	6.8 ± 1.3	nd	nd	nd

Table 3 shows that Pg 2 ϵ enhanced cellular invasion 6–7-fold. Co-incubation of Pg 2 ϵ with anti-u-PA or anti-MMP-9 antibodies, which inhibit enzymic activity, reduced invasiveness to nearly undetectable levels. Similar results were observed with cells co-incubated with Pg 2 ϵ and anti-DPP IV IgG. These results further demonstrate that Pg 2 ϵ is the only glycoform that significantly enhances 1-LN cell invasive activity, an effect resulting from its capacity to stimulate expression of MMP-9.

DISCUSSION

Degradation of ECM components occurs during a variety of tissue remodelling processes, including tumour invasion and rheumatoid arthritis. A complex mechanism requiring the fibrinolytic system and MMPs governs tumour stromal generation and the development of a vascular pannus in rheumatoid arthritis [55]. In both abnormalities, the Pg activation system and the production of MMPs are up-regulated, leading to the degradation of ECM components, which contributes to both articular destruction in rheumatoid arthritis and penetration of basement membranes by spreading cancer cells [55,56]. For these reasons, we investigated the possibility that similar Pg receptors also exist in human prostate cancer cells and that they are involved in the regulation of MMP-9 expression and activation. We studied the highly invasive 1-LN human prostate tumour cell line [57], because these cells synthesize and secrete large amounts of u-PA and MMPs [17]. Unlike rheumatoid synovial fibroblasts, we did not find the $\beta 3$ integrin associated with the membrane glycoprotein DPP IV. Our findings are summarized in Table 4. The less sialylated Pg 2 α and Pg 2 β glycoforms bind to these cells via L-lysine binding sites, they do not elicit a $[\text{Ca}^{2+}]_i$ response, and they are not involved in the secretion or expression of MMP-9. Pg 2 γ , Pg 2 δ and Pg 2 ϵ bind to DPP IV via their sialic acid residues and induce a $[\text{Ca}^{2+}]_i$ response [20]; however, only Pg 2 ϵ is able to induce expression and secretion of MMP-9. The $[\text{Ca}^{2+}]_i$ response in synovial fibroblasts requires binding of Pg to the integrin $\beta 3$ and activation by u-PA before their interaction with DPP IV [19,20], whereas in 1-LN cells a direct reaction of Pg with DPP IV induces a similar response. The identity of the L-lysine-dependent receptors of Pg 2 α and Pg 2 β on 1-LN cells

Table 4 Function of Pg 2 glycoforms on the surface of 1-LN cells

Glycoform	Binding		Increase in $[Ca^{2+}]_i$	MMP-9 expression	
	+6-AHA	+L-lactose		Secretion*	mRNA†
Pg 2 α	No	Yes	No	No	No
Pg 2 β	No	Yes	No	No	No
Pg 2 γ	Yes	No	Yes	No	No
Pg 2 δ	Yes	No	Yes	No	No
Pg 2 ϵ	Yes	No	Yes	Yes	Yes
Pg 2 ϕ	No binding				

* Data are shown in Table 2 and Figures 6 and 7.

† Data are shown in Figure 8.

remains unknown; however, due to its potential as a regulatory site, we are currently investigating this further.

In the circulation, the concentration of Pg 2 is 2-fold greater than that of Pg 1; however, in the extravascular space the concentration of Pg 2 is almost 6-fold greater than that of Pg 1 [58]. Pg 1 contains one O-glycan at Thr-345 and one biantennary N-glycan at Asn-288, whereas Pg 2 contains only the O-glycan chain [59,60]. Pg 1 activation is enhanced more than that of Pg 2 in the presence of fibrin by either u-PA or t-PA [61], suggesting a preferred role for Pg 1 in the intravascular space [58]. The shift in the ratio of Pg 2 to Pg 1 in the extravascular space suggests a significant role for Pg 2 glycoforms as the preferred forms for plasmin formation during metabolism on the cell surface [62]. In this context, Pg 2 ϵ should function preferentially at the cell surface, where its carbohydrate content, in general, and sialic acid, in particular, may play an important role in regulating its function.

Pg 2 δ and Pg 2 ϵ contain almost the same amount of sialic acid (5.77 and 5.34 mol of sialic acid/mol of Pg respectively). However, the pI of Pg 2 ϵ is more acidic [24], suggesting an additional secondary modification of its structure which may be critical for its capacity to induce the expression of MMP-9. This shift in the pI of Pg 2 ϵ may be associated with phosphorylation of the Pg molecule [63]. In this context, a shift in the pI of u-PA from 9.2 to 7.6, secondary to the phosphorylation of Tyr and Ser residues, is associated with the activation of pp60^{src} and of protein kinase C in metastatic tumour cells [64–66]. However, no data are available with respect to the kinases involved in the phosphorylation of Pg and u-PA, or its role in the multiple biological functions exerted by these proteins.

The Pg-mediated invasive activity of 1-LN cells is effectively blocked by mAbs which inhibit the enzymic activity of u-PA or MMP-9, suggesting that plasmin in the tumour cell micro-environment can enhance the invasive activity, either by direct proteolysis of ECM components [13,67] or via its capacity to activate proMMP-9 bound to a cell surface [5]. Recent studies in mice with targeted inactivation of the t-PA, u-PA or Pg genes [68] suggest that proMMP-9 activation may occur in the absence of t-PA or u-PA, whereas no active MMP-9 is detected in the absence of Pg. The mechanism whereby Pg is activated in this setting is unknown. Our studies demonstrate that Pg influences cell migration not only through its capacity to generate plasmin, which degrades fibrin, but also because it stimulates MMP-9 expression and activation.

In addition to the multiple functions that DPP IV performs on T cells, where it is known as CD26 [69], this glycoprotein is an endothelial cell adhesion molecule that mediates lung metastases by rat breast cancer cells [70]. Expression of MMP-2 and MMP-

9 by A2058 human melanoma cancer cells is also independently regulated by receptor-operated Ca^{2+} influxes, although no specific physiological ligand has been identified [23,71,72]. Our results provide new evidence connecting DPP IV with the Pg activation enzymic system and the expression of MMP-9, and suggest a biochemical mechanism by which Pg might regulate MMP-9 in the extracellular environment.

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