Human α_1-acid glycoprotein binds to CCR5 expressed on the plasma membrane of human primary macrophages

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We have reported previously that human α_1-acid glycoprotein (AGP) inhibits the infection of human monocyte-derived macrophages (MDM) by R5 HIV-1, and that a disulphide-bridged peptide mimicking the clade B HIV-1 gp120 consensus V3 domain (V3Cs) binds specifically to CCR5 (the major co-receptor of R5 HIV strains) on these cells [Seddiki, Rabehi, Benjouad, Saffar, Ferriere, Gluckman and Gattegno (1997) Glycobiology **7**, 1229–1236]. The present study demonstrates that AGP binds specifically to MDM at high- and low-affinity binding sites with K_d values of 16 nM and 4.9 μM respectively. The fact that heat denaturation of AGP only partly inhibited this binding (43 %) suggests that protein–protein interactions are involved, as well as AGP glycans which are resistant to heat denaturation. Mannan, but not dextran, is a significant inhibitor (52 %) of this binding, and sequential exoglycosidase treatment of AGP, which exposes penultimate mannose residues, has a strong stimulatory effect ($\sim$ 2.8-fold). Therefore AGP glycans (probably mannose residues) are involved, at least partly, in the binding of AGP to MDM. In addition, AGP inhibits the binding of V3Cs and macrophage inflammatory protein-1β (MIP-1β) to MDM. The anti-CCR5 monoclonal antibody 2D7, specific for the second extracellular loop of CCR5, also inhibited AGP binding (67 %), whereas anti-CCR5 antibodies specific for the C-terminus of CCR5 region had no effect. Native AGP, like V3Cs (but not heat-denatured AGP), binds to 46 and 33–36 kDa electroblotted AGP-bound MDM membrane ligands, characterized as CCR5 by their interactions with anti-CCR5 antibodies and with MIP-1β. Therefore both AGP glycans and MDM CCR5 are involved in the binding of AGP to MDM. This suggests that the inhibitory effect of AGP on the infection of human primary macrophages by R5 HIV-1 may be related to specific binding of AGP to a macrophage membrane lectin or lectin-like component and to CCR5.

Key words: glycan, HIV-1, lectin, monocyte-derived macrophages.

INTRODUCTION

CD4 is the primary receptor for HIV-1 [1], but other co-receptors have now been identified as chemokine receptors, in particular CCR5 for R5 HIV strains and CXCR4 for X4 HIV strains [2–6]. Because the HIV-1 envelope glycoprotein gp120 is highly glycosylated, it has been proposed that some of its glycans may be involved in viral infection [7–9], especially since lectin–carbohydrate interactions can mediate the attachment of micro-organisms to cells [10].

Because gp120 could interact with membrane lectins and/or with cell surface carbohydrate constituents, we previously examined [11] whether the natural glycoproteins bovine fetuin, its desialylated form asialofetuin, human α-fetoprotein and α_1-acid glycoprotein (AGP), and the polymannose compound mannan, which have been shown to interact specifically with gp120 via their mannose or GlcNAc residues [12,13], may compete with these interactions and therefore inhibit the infection of $CD4^+$ lymphocytes or macrophages by X4 or R5 HIV strains [14]. Physiological AGP and α-fetoprotein concentrations were used in these inhibition experiments [11]. We observed that bovine fetuin, asialofetuin, α-fetoprotein, AGP and mannan significantly inhibited the binding of gp120 to human primary macrophages, but not to monocytic U937 or lymphoid CEM cell lines [11]. This indicated that gp120 does not bind in the same manner to primary macrophages or to immortalized $CD4^+$ cells. We then showed that AGP interacts with and inhibits the binding of a disulphide-bridged peptide mimicking the clade B HIV-1 gp120 consensus V3 domain (V3Cs) to several macrophage membrane proteins, including a 46 kDa protein [11]. In addition, this peptide (V3Cs) binds to membrane proteins of human monocyte-derived macrophages (MDM) that comprise CD4 and CCR5 [15]. Finally, we demonstrated that bovine fetuin, bovine asialofetuin, human AGP, human α-fetoprotein and mannan, which interact specifically with gp120 [11], inhibited the infection of human primary macrophages by R5 HIV-1 *in vitro*, while dextran had no such effect. These data suggest that natural glycoproteins, such as AGP, may interfere with post-binding events during HIV-1 infection of primary macrophages [11]. However, these compounds did not modify the infection of U937 or CEM cells by X4 HIV-1_{LAI}, or the infection of peripheral blood lymphocytes by X4 HIV-1_{LAI} or R5 HIV-$1_{Ba\text{-}L}$ [11]. These results were related to differences in the expression of lectins at the membranes of lymphocytes and macrophages [11]. Thus the inhibition of HIV infection depended on the cell type more than on the viral strain.

The aim of the present study was to investigate how AGP inhibits the infection of human primary macrophages by R5 HIV-1. For this purpose, we have analysed the interactions of AGP with human MDM, and determined whether this glycoprotein competes with macrophage inflammatory protein-1β (MIP-1β), the most specific CCR5 ligand [2], for binding to these

Abbreviations used: AGP, α_1-acid glycoprotein; ECL2, second extracellular loop of CCR5; mAb, monoclonal antibody; MDM, monocyte-derived macrophages; MIP-1β, macrophage inflammatory protein-1β; SDF-1α, stromal-cell-derived factor-1α; V3Cs, disulphide-bridged peptide mimicking the clade B HIV-1 gp120 consensus V3 domain.

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cells, and whether it interacts specifically (like V3Cs and MIP-1β) with the HIV-1 co-receptor CCR5. As AGP is characterized by a high carbohydrate content (42 %), with five linked bi-, tri- or tetra-antennary glycans per molecule [16,17], we have also investigated the possible role of AGP glycans in the binding of AGP to MDM.

EXPERIMENTAL

Glycoproteins, chemokines and peptides

AGP was from Sigma-Aldrich (Saint Quentin Fallavier, France). The purity of intact or iodide-labelled AGP was > 99 %, as assessed by SDS/PAGE (12 %) on mini gels (Novex, San Diego, CA, U.S.A.), followed by Coomassie Blue staining (Bio-Rad Laboratories, Richmond, VA, U.S.A.) or autoradiography. Chicken egg ovalbumin and transferrin were from Sigma-Aldrich. Synthetic stromal-cell-derived factor-1α (SDF-1α) was a gift from F. Baleux (Institut Pasteur, Paris, France). Recombinant MIP-1β was from R&D Systems (Abingdon, Oxon., U.K.).

V3Cs (CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQA-HC), a peptide with a disulphide bridge between the two Cys residues, was from Neosystem (Strasbourg, France).

Labelling of AGP, V3Cs and chemokines

AGP, V3Cs, MIP-1β and SDF-1α were radiolabelled with 0.2 mCi of Na^{125}I (Amersham, Paris, France) using Iodobeads (Pierce Europe, BA Oud-Beijerland, The Netherlands) according to the manufacturer's instructions. Unreacted reagents were gel-filtered on PD10 Sephadex G-25M columns (Pharmacia Biotech, Orsay, France). Specific radioactivities were 0.1–0.4 MBq/μg for AGP and V3Cs, 0.2–0.8 MBq/μg for SDF-1α and 1.5–2.5 MBq/μg for MIP-1β. The labelled chemokines were submitted to SDS/PAGE (16 % gels) and characterized in accordance with our previous study by their apparent molecular mass of 8 kDa [18]. ^{125}I-labelled AGP was characterized on SDS/PAGE (12 % gels) by its molecular mass of 42 kDa (results not shown).

Cells

Blood samples were obtained from healthy HIV-seronegative donors (Seine-Saint-Denis Blood Transfusion Center, Bobigny, France). Peripheral blood mononuclear cells (from cytapheresis) were separated by Ficoll–Hypaque gradient density centrifugation. These cells were cultured for 5 days in RPMI 1640, 50 μg/ml penicillin/streptomycin, 2 mM L-glutamine (Gibco-BRL, Paisley, Scotland, U.K.) and 20 % (v/v) fetal calf serum (Boehringer Mannheim) (R20) supplemented with 10 % (v/v) heat-inactivated normal human pooled AB serum (Seine-Saint-Denis Blood Transfusion Center) [19–21]. Non-adherent cells were then removed by several washes in Ca^{2+}- and Mg^{2+}-free PBS. Adherent cells were cultured in the same medium without AB serum but with 20 % (v/v) fetal calf serum (R20) for another 24–48 h before they were exposed to ligands or scraped off with a rubber policeman. This procedure yielded > 90 % CD14$^+$ viable MDM which expressed membrane CD4, as reported previously [19–21]. No contaminating T cells were noted under these conditions (results not shown).

Flow cytofluorimetric analysis

MDM were scraped off with a rubber policeman. Portions of 5×10^5 MDM were washed twice with PBS, and then incubated for 30 min at 4 °C in 100 μl of PBS containing 0.05 % (w/v) BSA (Sigma-Aldrich), supplemented or not with 0.05 % sodium azide (Sigma-Aldrich), and with or without 1 μg of FITC-labelled anti-CD4 monoclonal antibody (mAb) (clone 13B.8.2; Immunotech, Marseille, France), anti-CD14 mAb or anti-CD3 mAb (Becton Dickinson, Mountain View, CA, U.S.A.), or their isotype controls, IgG1 (Immunotech) or IgG2b (Becton Dickinson). Cells were incubated in parallel with 2.5 μg of anti-CCR5 mAb 2D7 or 2.5 μg of anti-CXCR4 mAb 12G5 (both from Pharmingen, San Diego, CA, U.S.A.) or their isotype control, IgG2a (Sigma-Aldrich). After washing, cells were incubated for 30 min at 4 °C with FITC-labelled goat anti-(mouse IgG) antibodies (1:20; Coulter Coultronics, Roissy, France). After three washes at 4 °C, cells were fixed in 1 % paraformaldehyde (Sigma-Aldrich) in PBS and analysed by flow cytometry on a FACScan instrument (Becton Dickinson).

In some experiments, the MDM were preincubated for 30 min at 4 °C with AGP (up to 20 μM), and then co-incubated with the antibodies as decribed above.

Specific binding of AGP and MIP-1β to MDM

Approx. 5×10^5 MDM were scraped off, washed three times in RPMI and incubated for 1 h at 4 °C in RPMI supplemented with 0.05 % BSA (RPMI/BSA) and 30–50 nM ^{125}I-AGP [$(3–8) \times 10^4$ c.p.m.] or 1–3 nM ^{125}I-MIP-1β or ^{125}I-SDF-1α [both $(3–8) \times 10^4$ c.p.m.], with or without a 10–1000-fold molar excess of non-labelled AGP, MIP-1β or ovalbumin, or 0–330 μg/ml of mannan (from *Saccharomyces cerevisiae*) or dextran (both from Sigma-Aldrich). Values for the displacement of binding of ^{125}I-AGP or ^{125}I-MIP-1β to MDM by some of these compounds were analysed by fitting to a logistic curve or by the method of Scatchard. The logistic curve took the form:

$$\text{Binding (\% of max.)} = \text{msb}/[1 + 10^{(\log IC_{50} - \log C)}]$$

where C is the compound concentration, and msb, the maximum specific binding, is the difference between the maximum binding observed at the lowest ligand concentration and the minimum binding observed at the highest ligand concentration. Non-specific binding of ^{125}I-AGP or ^{125}I-MIP-1β to MDM was determined in the presence of 1–10 μM unlabelled AGP or MIP-1β. Results are expressed as means $\pm$ S.D. of three to four independent assays.

In some experiments, cells were preincubated with mAbs or polyclonal antibodies: up to 83 μg/ml anti-CCR5 C-20 antibodies (Tebu; Santa Cruz Biotechnology), up to 83 μg/ml anti-CCR5 2D7 mAbs, up to 83 μg/ml anti-CXCR4 12G5 mAbs or up to 40 μg/ml anti-CD4 Q4120 mAbs (Sigma-Aldrich), or the respective isotype controls (goat IgG from Tebu; rabbit IgG2b or mouse IgG1 from Sigma-Aldrich). In some experiments, ^{125}I-AGP was heat-treated for 10 min at 100 °C or pretreated for 1 h at 37 °C with neuraminidase (3 m-units), followed or not by a 1 h treatment with β-galactosidase (3 m-units), and in some assays by an additional 1 h treatment with *N*-acetyl-β-D-glucosaminidase (3 m-units) (all from Boehringer Mannheim or Glyko, Novato, CA, U.S.A.). MDM were then washed three times with RPMI-BSA, and radioactivity was counted in a Beckman γ-radiation counter. Enzyme efficiency was controlled using SDS/PAGE (12 % gels) by determination of the molecular mass of AGP before and after enzyme treatment (results not shown).

In other experiments, binding of ^{125}I-V3Cs (0.25 μM) to MDM was studied in the presence of a 0–100-fold molar excess of unlabelled AGP or transferrin (used as a control). Alter-

natively, binding of ^{125}I-AGP to MDM was also performed in the presence of a 100-fold molar excess of unlabelled V3Cs. We have reported previously that total binding of ^{125}I-V3Cs (0.1–0.5 μM) to MDM was displaced by 80 % and 90 % by a 10-fold molar excess of unlabelled V3Cs and by rabbit anti-V3Cs serum (Neosystem) respectively [15].

Binding of AGP to CCR5

Washed and scraped MDM [(1–2) $\times$ 10^7] were incubated for 3 h at 4 °C in 0.5 ml of PBS/BSA, with or without 0.19 μM AGP. A temperature of 4 °C was used to avoid receptor internalization, which may occur at 37 °C [22]. Cells were washed three times with PBS and lysed for 30 min at 4 °C in 500 μl of lysis buffer (150 mM NaCl, 20 mM Tris/HCl, pH 8.2, supplemented with 1 % Brij 97, 1 mM PMSF, 0.25 mM phenanthroline and 20 μg/ml aprotinin; all from Sigma-Aldrich). Lysates were centrifuged at 1000 ***g*** for 30 min at 4 °C, and immunoprecipitated by incubation for 18 h at 4 °C with 100 μl of Protein A–agarose beads (EY Laboratories, San Mateo, CA, U.S.A.) precoated or not with either 2.5 μg of polyclonal anti-AGP antibodies (Sigma-Aldrich) or 2.5 μg of rabbit control IgG. Binding of antibodies to beads was performed for 2 h at 20 °C, and beads were washed exhaustively in buffer. Beads were then boiled for 10 min with 120–150 μl of 2 $\times$ Laemmli sample buffer and centrifuged (400 ***g*** for 10 min at 15 °C); supernatants were submitted to SDS/PAGE (12 % polyacrylamide) under reducing conditions and transferred on to Immobilon strips, which were then saturated for 2–18 h at 37 °C with PBS/BSA. Excess BSA was washed out with PBS containing 0.5 % BSA and 0.2 % Tween 20 (Sigma), and strips were incubated for 18 h at 37 °C with either native or heat-treated (10 min at 100 °C in the presence of 1 % SDS and 5 % β-mercaptoethanol) ^{125}I-AGP, ^{125}I-V3Cs, ^{125}I-MIP-1β or ^{125}I-SDF-1α (each at approx. 2 nM; 10^6 c.p.m.).

In some experiments, ^{125}I-AGP was preincubated for 1 h at 37 °C with 1 mg/ml mannan or dextran; mixtures were then incubated with the strips as described above. In other experiments, 20 μg of ^{125}I-AGP was pretreated for 18 h at 37 °C with 10 m-units of N-Glycanase (peptide N-glycosidase F from *Flavobacterium meningosepticum*; Glyko) in 20 mM sodium phosphate, pH 7.5, containing 50 mM EDTA and 0.05 % sodium azide. The efficiency of N-Glycanase was analysed by the determination of the molecular mass of intact and N-Glycanase-treated AGP, as assessed by SDS/PAGE (12 % polyacrylamide), followed by autoradiography or Coomassie Blue staining.

After six washes with the buffer, strips were exposed at −20 °C for 18–72 h. Alternatively, strips were saturated for 18 h at 37 °C with 20 mM Tris-buffered saline, pH 7.6, and washed three times in Tris-buffered saline supplemented with 0.5 % (w/v) BSA and 0.1 % (v/v) Tween. Strips were then incubated for 1 h at room temperature with goat polyclonal anti-CCR5 antibodies or mouse anti-CCR5 mAb 2D7, or their respective isotype controls (at 1:1000). After three washes, strips were incubated with anti-goat or anti-mouse peroxidase-labelled IgG (at 1:5000). Strips were washed three times and revealed by enhanced chemiluminescence reagent (Amersham).

RESULTS

Binding characteristics for ^{125}I-AGP on MDM

In the present study, no exogeneous cytokine was added, but the MDM were first cultured in the presence of autologous lymphocytes, which may release endogenous cytokines, before the lymphocytes were carefully washed away. These MDM expressed CD14, CD4, CCR5 and CXCR4, as assessed by cytofluorimetric analysis after immunofluorescence labelling (Figure 1, Table 1).

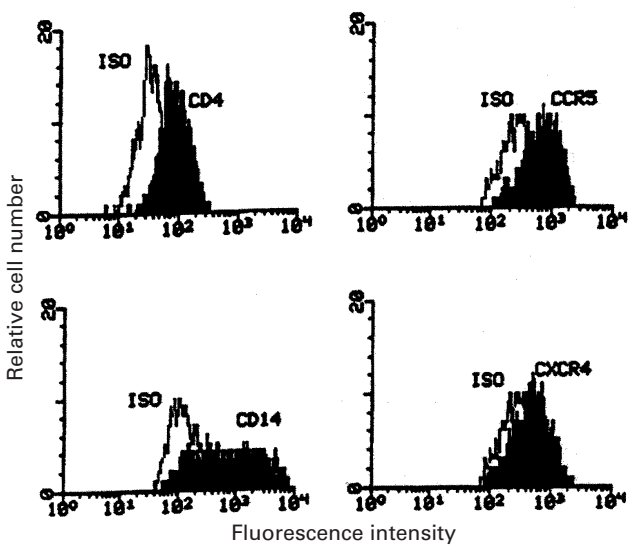

Figure 1 MDM membrane expression of CD4, CD14, and the chemokine receptors CCR5 and CXCR4

MDM after 7 days in culture were labelled with mAbs against CD4, CD14, CCR5 or CXCR4 (black peaks), or with isotype control antibodies (white peaks; ISO). Data are representative of at least eight experiments using blood from different donors.

Table 1 MDM membrane expression of CD4, CD14 and chemokine receptors CCR5 and CXCR4 in the absence or the presence of AGP

'FITC-' indicates labelling by FITC. The results are representative of three to eight independent experiments. ND, not done.

	Mean fluorescence intensity (units)	
Antibodies used	−AGP	+AGP
FITC-IgG1	39	39
FITC-anti-CD4 mAb (clone 13B.8.23)	108	108
IgG2b + FITC-anti-(mouse IgG) goat antibodies	200	ND
Anti-CD14 mAb + FITC-anti-(mouse IgG) goat antibodies	1406	ND
IgG2a + FITC-anti-(mouse IgG) goat antibodies	378	378
Anti-CCR5 mAb 2D7 + FITC-anti-(mouse IgG) goat antibodies	702	702
Anti-CXCR4 mAb 12G5 + FITC-anti-(mouse IgG) goat antibodies	590	590

^{125}I-AGP bound in a dose-dependent manner to the MDM; this binding was significantly inhibited by increasing concentrations of unlabelled AGP, but not by ovalbumin, another glycoprotein used as control (Figure 2; and results not shown). Non-specific binding of ^{125}I-AGP to MDM, determined in the presence of 10 μM AGP, was a mean of 7 % of total AGP binding (Figure 2). Scatchard analysis of the displacement curve, derived from four independent assays of ^{125}I-AGP (30 nM) binding to MDM in the presence of increasing concentrations of unlabelled AGP (up to 10 μM), revealed two classes of specific

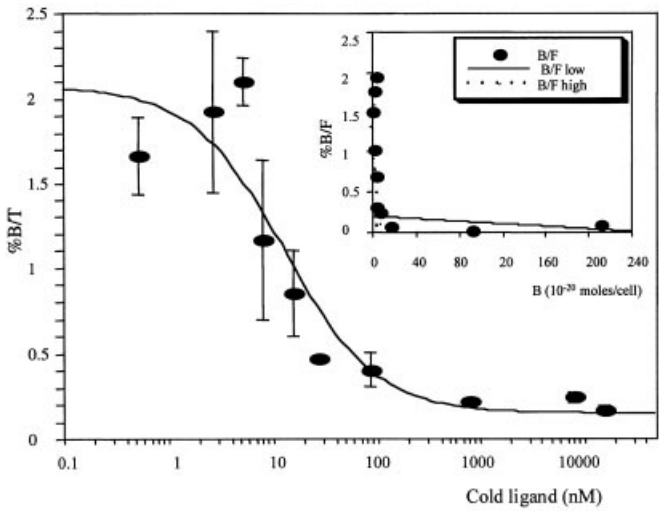

Figure 2 Characteristics of binding of ^{125}I-AGP to MDM

The displacement curve shows the inhibition of the binding of ^{125}I-AGP (30 nM) to MDM by increasing concentrations of unlabelled ('cold') AGP. Data are means ± S.D. of four independent assays, each performed in duplicate or triplicate. The inset presents data transformed by Scatchard analysis. Scatchard analysis of ^{125}I-AGP binding revealed two K_d values: one for high-affinity binding sites and the other for low-affinity binding sites. B, bound; T, total; F, free.

binding sites: one class with 43000 ± 13000 binding sites per cell and a K_d of 16 ± 1.57 nM, and the other with 15600000 ± 560000 binding sites per cell and a K_d of 4.9 ± 0.57 μM (Figure 2).

However, preincubation of MDM with AGP (up to 20 μM) did not modify the binding of anti-CCR5 2D7 or anti-CD4 mAbs, which indicates that the AGP binding sites on MDM differ from the epitopes recognized by the anti-CCR5 2D7 and anti-CD4 mAbs (Table 1).

Involvement of AGP glycans and CCR5 of MDM in the binding of AGP to MDM

Heat denaturation of AGP resulted in a significant, but partial, inhibition of the total binding of ^{125}I-AGP to MDM (43 ± 13 % inhibition; $P < 0.02$; $n = 3$) (Figure 3a). This demonstrates that the three-dimensional structure of ^{125}I-AGP is only partly responsible for this binding, and suggests that, in addition to protein–protein interactions, AGP glycans that are resistant to heat denaturation may play a role. Indeed, total binding of ^{125}I-AGP to MDM was also partially but significantly displaced by mannan at 330 μg/ml (52 ± 4 % inhibition; $P < 0.001$; $n = 4$), while dextran had no effect (Figure 3a). Interestingly, mannans from a variety of sources are reported to be good ligands for mannose-binding proteins, such as the human mannose receptor, which is present on many tissue-differentiated macrophages and on MDM, but not on freshly isolated peripheral blood monocytes [23]. To analyse further the role of AGP glycans in this binding, ^{125}I-AGP was submitted to exoglycosidase treatments: while removal of sialic acid from ^{125}I-AGP by neuraminidase had no significant effect ($n = 5$), sequential treatment of ^{125}I-AGP with neuraminidase and β-D-galactosidase slightly, but significantly, enhanced ^{125}I-AGP binding ($n = 3$; $P < 0.01$). However, when *N*-acetyl-β-D-glucosaminidase was added after these two enzyme treatments, a marked and significant increase (approx. 2.8-fold) in binding was observed ($n = 3$; $P < 0.02$); this may be related to exposure of penultimate mannose residues (Figure 3b). These results indicate strongly that AGP glycans, probably mannose residues, are involved in the binding of AGP to MDM, and rule out a role for the sialic acid residues of AGP. Pretreatment of AGP with N-Glycanase F also significantly enhanced (approx. 2.5-fold; $n = 7$; $P < 0.05$; results not shown) the binding of AGP to MDM; however, this result may be related to the incomplete deglycosylation of AGP by this enzyme treatment under non-denaturating conditions, leading also to the exposure of mannose residues. Indeed, whereas the molecular mass of intact AGP was 42 kDa, as assessed by SDS/PAGE (12 % polyacrylamide), the N-Glycanase F-treated AGP preparations were characterized by several proteins with molecular masses of 36, 32 and 29 kDa, which indicates the presence of several incompletely deglycosylated AGP molecules (results not shown). Taken together, our data demonstrate that AGP interacts specifically with human MDM, and indicate the involvement of both protein–protein interactions and glycans.

In accordance with our previous data [11], the total binding of ^{125}I-V3Cs to MDM was inhibited significantly by unlabelled AGP. Maximum inhibition was achieved in the presence of a 10-fold molar excess of unlabelled AGP (43 ± 15 % inhibition; $n = 3$; $P < 0.01$), but was not affected by transferrin used as control (Figure 3c). Increasing the amount of unlabelled AGP up to a 100-fold molar excess did not modify this inhibition. Moreover, total binding of ^{125}I-AGP to MDM was also significantly, but slightly, inhibited by unlabelled V3Cs present at up to a 100-fold molar excess (25 ± 10 % inhibition; $n = 3$; $P < 0.05$; results not shown). Taken together, these results indicate that V3Cs and AGP, of molecular masses 3.8 kDa and 42 kDa respectively, may bind to MDM membrane components that are spatially close to each other, but not similar. Since we have shown previously a specific interaction between V3Cs and CCR5 [15], we next analysed, at the cellular level, whether CCR5 may be involved in the binding of AGP to MDM. This binding was decreased significantly by the anti-CCR5 mAb 2D7, which is specific for a conformational domain located in the second extracellular loop of CCR5 (ECL2). The anti-CCR5 mAb 2D7 at 500 nM displaced 67 ± 4 % of the total binding of ^{125}I-AGP to MDM as compared with that observed in the presence of the isotype control ($P < 0.05$; $n = 3$) (Figure 3d); this displacement had an IC_{50} of 150 nM. In contrast, polyclonal anti-CCR5 antibodies (C-20), specific for the C-terminal region of CCR5, had no effect at concentrations up to 500 nM (Figure 3d). Preincubation of MDM with AGP did not modify the binding of the anti-CCR5 mAb 2D7 (Table 1). Therefore AGP does not attach to a site on CCR5 that is required for binding of the anti-CCR5 mAb 2D7; thus mAb 2D7 may exert its inhibitory effect on AGP binding to CCR5 by altering the conformation of CCR5 [24], or by steric hindrance of the interaction of AGP with another region of the receptor.

The anti-CD4 mAb Q4120, which is specific for the HIV gp120 binding domain of CD4, and the anti-CXCR4 mAb 12G5, at concentrations up to 500 nM, had only slight (< 25 %) inhibitory effects on the binding of ^{125}I-AGP to MDM (mean inhibition of 18 ± 8 % and 23 ± 2.5 % respectively as compared with binding observed in the presence of the respective isotype controls; $P < 0.05$; $n = 4$) (Figure 3d).

Since it has been demonstrated previously that MIP-1β is the most specific ligand for CCR5 [2], we next investigated whether AGP is able to displace binding of this chemokine to MDM. For this purpose, we first analysed the characteristics of binding of ^{125}I-MIP-1β to the MDM; as expected, ^{125}I-MIP-1β bound specifically to the MDM, which express CCR5, glycans and glycosaminoglycans [25]. Non-specific binding of ^{125}I-MIP-1β to MDM, determined in the presence of 1–10 μM unlabelled

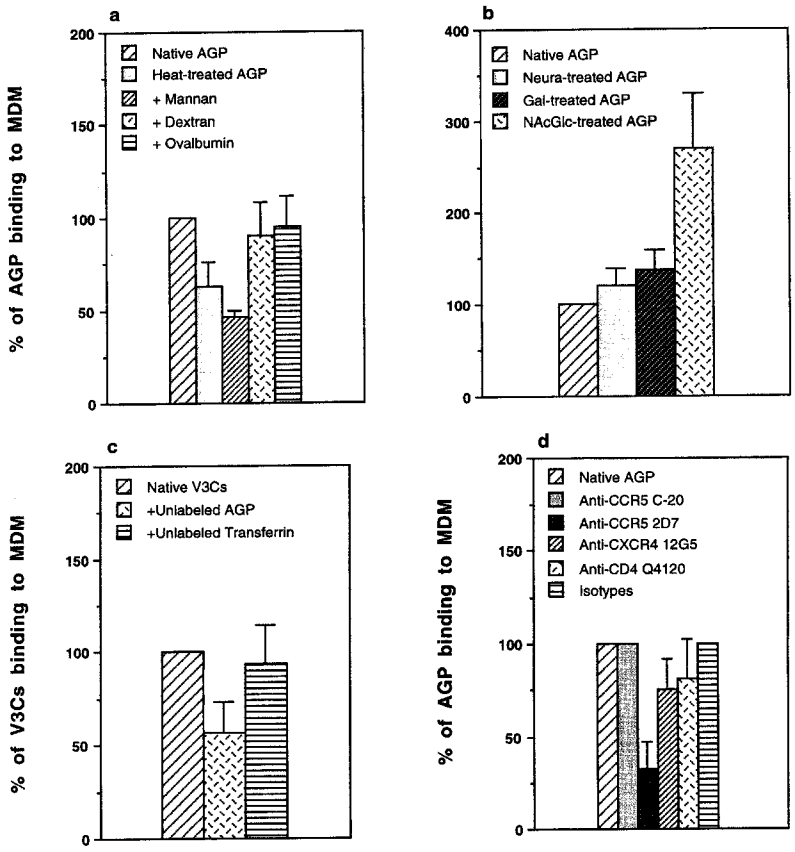

Figure 3 Involvement of AGP glycans and MDM CCR5 in binding of AGP to MDM

(**a**) Total binding of native ^{125}I-AGP (30 nM) or heat-treated ^{125}I-AGP (30 nM) to MDM. The statistical significance of differences was determined by the paired Student's *t* test ($P < 0.02$; $n = 3$). Also shown is the displacement of binding of native ^{125}I-AGP to MDM by 330 μg/ml mannan ($P < 0.001$; $n = 4$), but not by 330 μg/ml dextran or ovalbumin. (**b**) Total binding to MDM of intact ^{125}I-AGP and of ^{125}I-AGP treated with neuraminidase alone (Neura-treated AGP), with neuraminidase plus β-galactosidase (Gal-treated AGP), or with neuraminidase, β-galactosidase and *N*-acetyl-β-D-glucosaminidase (NAcGlc-treated AGP). The significance of coupled differences between the binding of intact AGP and that of treated AGP was as follows: neuraminidase, not significant ($n = 5$); neuraminidase plus β-galactosidase, $P < 0.01$ ($n = 3$); neuraminidase, β-galactosidase and *N*-acetyl-β-D-glucosaminidase, $P < 0.02$ ($n = 3$). (**c**) Displacement of total binding of ^{125}I-V3Cs (0.25 μM) to MDM by unlabelled AGP (2.5 μM) ($P < 0.01$; $n = 3$), but not by transferrin (2.5 μM). Unlabelled V3Cs (2.5 μM) displaced a mean of 80% of the total binding of ^{125}I-V3Cs to MDM. Results are expressed as means $\pm$ S.D. of three independent experiments. (**d**) Inhibition of total binding of native ^{125}I-AGP to MDM by anti-CCR5 mAb (2D7) ($P < 0.05$; $n = 3$) and by anti-CXCR4 (12G5) or anti-CD4 (Q4120) mAbs ($P < 0.05$; $n = 4$), but not by polyclonal anti-CCR5 antibodies (C-20) or by their respective isotype controls (Isotypes). Non-specific binding of ^{125}I-AGP (30 nM) to MDM, determined in the presence of 10 μM unlabelled AGP, was a mean of 7% of total ^{125}I-AGP binding. Results are expressed as means $\pm$ S.D. of three to four independent experiments.

MIP-1β, represented a mean of 10% of total binding. Scatchard analysis of the values derived from the displacement curves ($n = 3$) for binding of ^{125}I-MIP-1β (1–3 nM) to MDM in the presence of increasing concentrations of unlabelled MIP-1β (up to 10 μM) indicated the presence of 7000 ± 2000 high-affinity binding sites per MDM, characterized by a K_d of 0.5 ± 0.04 nM (Figure 4). This K_d is in good agreement with values reported for binding of chemokines to 7-TM receptors [26]. However, lower-affinity binding sites were also detected, at a density of $38\,000 \pm 11\,000$ per cell and characterized by a K_d of 10 ± 1.2 nM (Figure 4). Finally, we observed that binding of ^{125}I-MIP-1β (1–3 nM) to MDM was also strongly inhibited by increasing concentrations of unlabelled AGP (up to 5 μM); the displacement curve revealed an IC_{50} of 9.8 nM (Figure 5). Under the same experimental conditions, binding of ^{125}I-SDF-1α (1–3 nM) to MDM was not inhibited by AGP up to 8 μM (results not shown). Taken together, these results suggest that AGP and MIP-1β bind to close or similar membrane components of MDM, including CCR5, and strongly indicate that the HIV co-receptor CCR5, an HIV-1 gp120 V3Cs ligand [15], is involved in the binding of AGP to MDM.

Specific interaction of AGP with CCR5

Finally, we investigated at the molecular level whether AGP binds directly to CCR5 expressed on the MDM cell membrane. We have shown previously that, after electroblotting of nucleus-depleted MDM lysates on to Immobilon strips, biotinylated AGP bound to several ligands [11]. In the present study, non-labelled AGP was incubated with intact MDM, which were then lysed. The AGP-bound proteins were then immunoprecipitated by Protein A–agarose beads coated with anti-AGP antibodies. The AGP-bound and unbound proteins were analysed after SDS/PAGE and electrotransfer. We found that membrane proteins of 33–36 kDa and 46 kDa were retained by the anti-AGP-antibody-coated beads, which interacted with native ^{125}I-AGP and native ^{125}I-MIP-1β (Figure 6a, lanes 1 and 3), but not with their heat-denatured counterparts (Figure 6a, lanes 2 and

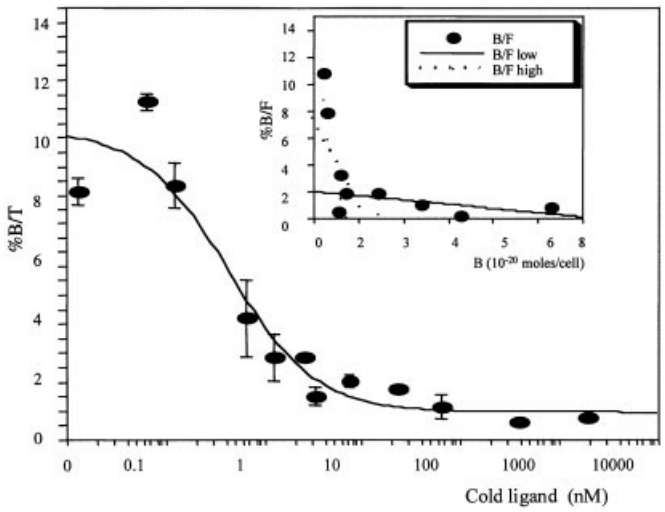

Figure 4 Characteristics of binding of ^{125}I-MIP-1β on MDM

Shown is the displacement curve for the binding of ^{125}I-MIP-1β (1 nM) to MDM in the presence of unlabelled ('cold') MIP-1β. Results are represented as means $\pm$ S.D. of three independent assays, each performed in duplicate or triplicate. The inset presents data transformed by Scatchard analysis. Scatchard analysis of ^{125}I-MIP-1β binding revealed two K_d values: one for high-affinity binding sites and the other for low-affinity binding sites. B, bound; T, total; F, free.

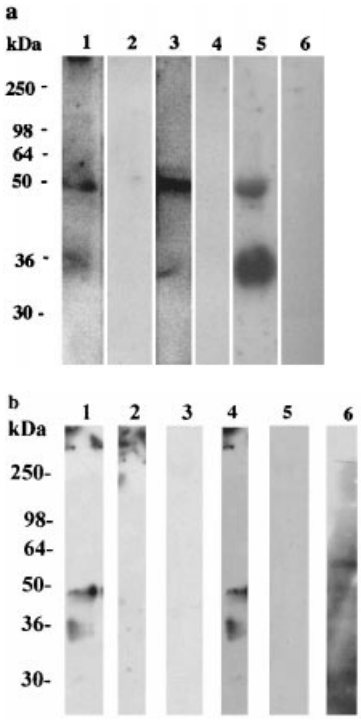

Figure 6 Detection of AGP-binding proteins on MDM

(**a**) Specific interaction of ^{125}I-AGP with AGP-binding proteins on MDM. Cells were incubated with AGP and lysed. Lysates were incubated overnight with Protein A–agarose beads pre-coated with rabbit anti-AGP polyclonal antibodies. The AGP-interacting retained material was electrotransferred to Immobilon strips and incubated with native ^{125}I-AGP (lane 1), heat-denatured ^{125}I-AGP (lane 2), native ^{125}I-MIP-1β (lane 3), heat-denatured ^{125}I-MIP-1β (lane 4), ^{125}I-V3Cs (lane 5) or ^{125}I-SDF-1α (lane 6). (**b**) Cells were treated as in (**a**), and the AGP-interacting retained material was incubated with anti-CCR5 antibodies (C-20) (lane 1) or with anti-CCR5 mAb 2D7 (lane 4), or the respective isotype controls (lanes 2 and 5). The AGP-unretained material was submitted to SDS/PAGE, transferred to Immobilon strips and incubated with anti-CCR5 (C-20) antibodies (lane 3) or with anti-CCR5 mAb 2D7 (lane 6). Results are representative of two or three independent experiments.

4). These results demonstrate that the three-dimensional structure of AGP is involved in its binding to these proteins, and strongly suggest the occurrence of protein–protein interactions. Indeed, preincubation of AGP with mannan followed by co-incubation of the mixture with the electroblotted macrophage membrane proteins, or pretreatment of AGP with N-Glycanase F, did not modify binding of AGP to these electroblotted proteins (results not shown), which suggests that AGP mannose residues are not involved. These membrane proteins of 33–36 kDa and 46 kDa,

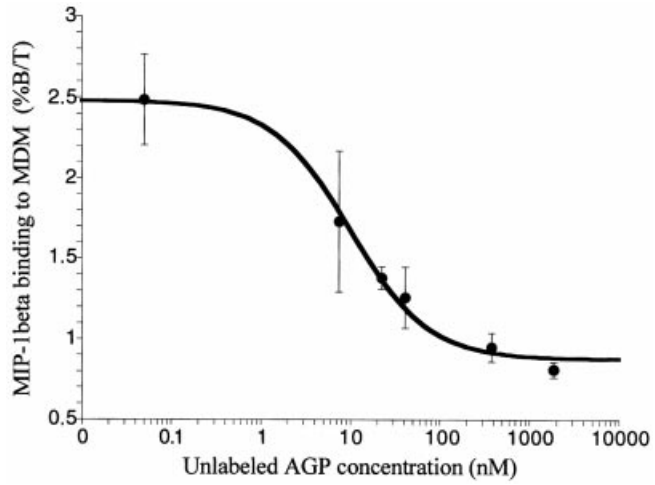

Figure 5 Displacement curve for binding of ^{125}I-MIP-1β to MDM in the presence of unlabelled AGP

This displacement curve for binding of ^{125}I-MIP-1β (1 nM) to MDM in the presence of unlabelled AGP reveals an IC_{50} of 9.8 nM. Results are represented as means $\pm$ S.D. of three independent assays, each performed in duplicate or triplicate.

which also interacted with ^{125}I-V3Cs (Figure 6a, lane 5), but not with ^{125}I-SDF-1α used as a negative control (Figure 6a, lane 6), were characterized further by their binding to anti-CCR5 polyclonal antibodies specific for the C-terminal domain of CCR5, as well as to the anti-CCR5 mAb 2D7, which is specific for a conformational domain located at ECL2, but not to the respective isotype controls (Figure 6b, lanes 1, 2, 4 and 5). No interaction of the AGP-unbound proteins with the anti-CCR5 polyclonal antibodies was observed, which indicates that, under our experimental conditions, all the CCR5 molecules that were immunoreactive with these antibodies have bound to AGP (Figure 6b, lane 3). However, among the AGP-unbound proteins, a 60 kDa protein was immunoreactive with the anti-CCR5 mAb 2D7 (Figure 6b, lane 6). Taken together, these data demonstrate that AGP binds to some, but not all, of the CCR5 molecules expressed at the MDM membrane, via protein–protein interactions.

DISCUSSION

AGP, a plasma glycoprotein belonging to the group of acute-phase proteins, contains five N-linked glycans which, depending on pathophysiological state, differ in their degree of branching,

i.e. in the relative proportions of di-, tri- and tetra-antennary glycans. Changes in the degree of branching of these glycans have been shown to affect various immunomodulatory properties of AGP [16]. Our previous data have shown that physiological AGP concentrations inhibit the infection of human MDM by R5 HIV-1 *in vitro*, and that AGP binds to several MDM ligands [11]. These observations therefore suggested that AGP may interfere with post-binding events during R5 HIV-1 infection of human primary macrophages [11]. The present study was thus designed to determine how AGP inhibits the infection of macrophages by R5 HIV-1.

We demonstrate here that AGP binds specifically to human MDM, which contain low- and high-affinity binding sites with B_{max} values of 43000 and 15600000 receptors per cell respectively and K_d values of 16 nM and 4.9 μM respectively. The fact that heat denaturation of AGP partially inhibited its binding to MDM indicates that the three-dimensional structure of AGP is involved in this binding, at least in part. These results strongly suggest that, in addition to the occurrence of protein–protein interactions, AGP glycans (which are resistant to heat treatment) are also involved in binding. Indeed, mannan significantly inhibits the binding of AGP to MDM, while dextran has no effect; furthermore, sequential exoglycosidase treatment of AGP, which removes sialic acid and galactose resulting in the exposure of penultimate mannose, induces a marked increase in binding of AGP to MDM. This further indicates that AGP glycans, probably mannose residues, may be involved, at least partly, in binding of AGP to MDM. Therefore the low-affinity binding of AGP to MDM may involve lectin–carbohydrate interactions through AGP glycans and some macrophage membrane lectin or lectin-like component, such as those described previously by others [23], while the high-affinity binding sites may be related to protein–protein interactions of AGP and MDM membrane components. We have shown previously that AGP and mannan significantly inhibited the binding of gp120 to human primary macrophages [11], suggesting that gp120 may interact with macrophage membrane lectins [11]. Therefore our previous and present data suggest that AGP glycans and gp120 glycans, which represent respectively approx. 42 % and 50 % of the molecular masses of these molecules, may bind to similar or spatially close macrophage membrane lectins or lectin-like components. Ly and Stamatatos [27] have recently observed that while the pattern of HIV or SIV (simian immunodeficiency virus) envelope glycosylation does not significantly affect viral replication ability in peripheral blood mononuclear cells, V2 loop glycosylation profoundly affects the ability of HIV to replicate in primary macrophages characterized by low levels of CD4 and CCR5. Therefore HIV envelope glycosylation appears to play an important role during envelope interaction with both CD4 and CCR5 at the membrane of primary macrophages [27].

In the present study, we observed that a mean of 43 % of the binding of V3Cs to MDM was inhibited by AGP, whereas a mean of 23 % of the binding of AGP to MDM was inhibited by V3Cs. Since the molecular mass of AGP is more than ten times that of V3Cs, these results indicate that V3Cs and AGP bind to spatially close, but not similar, macrophage membrane components. Although several HIV-1 gp120 domains are likely to be involved in the interactions of HIV-1 gp120 with CCR5 [27], V3Cs mimics one of the HIV-1 envelope regions that binds directly to CCR5 [15]; we therefore analysed at the cellular level whether AGP also binds CCR5. For this purpose, since MIP-1β is the the most specific CCR5 ligand [2], we investigated the characteristics of MIP-1β binding to MDM. Scatchard analysis revealed a mean of 7000 high-affinity specific binding sites per MDM, with a K_d of 0.5 nM; these data are in good agreement with values reported for the binding of chemokines to 7-TM receptors [26]. However, the existence of lower-affinity specific binding sites, with a B_{max} of 38000 per MDM and a K_d of 10 nM, is described here for the first time. These receptors may be involved in binding of MIP-1β to MDM molecules other than CCR5, such as glycosaminoglycans. Interactions of MIP-1β with glycosaminoglycans has been described previously, for instance by the use of CHO cell lines mutated to have a defect in normal glycosaminoglycans [26,28].

Interestingly, we observed that AGP is a strong inhibitor of the binding of MIP-1β, but not of SDF-1α, to the MDM, with an IC_{50} of 9.8 nM; this suggests the occurrence of competition between AGP and MIP-1β for binding to MDM components, including CCR5, and suggests that AGP and MIP-1β bind to close or similar CCR5 domains. Moreover, the anti-CCR5 mAb 2D7, which is specific for a CCR5 conformational domain located on ECL2 and known to be involved in the binding of HIV-1 gp120 and MIP-1β [24], significantly inhibited binding of AGP to MDM, whereas anti-CCR5 polyclonal antibodies specific for CCR5 C-terminal region had no effect. Taken together, these data indicate that AGP binds to CCR5. mAbs against ECL2 are potent inhibitors of HIV-1 infection [24]; mAb 2D7, which binds to an antigenic determinant in the first half of ECL2, is unique in its ability to block both chemokine and gp120 binding, as well as HIV-1 co-receptor activity [24]. However, preincubation of MDM with AGP does not modify the binding of the anti-CCR5 mAb 2D7. Therefore AGP does not attach to a site of CCR5 required for anti-CCR5 mAb 2D7 binding, and the latter may exert its inhibitory effect on AGP binding to CCR5 by altering the conformation of CCR5 [24] or by steric interference with the interaction of AGP with another region of the receptor. Indeed, the molecular mass of the anti-CCR5 mAb 2D7 (150 kDa) is about three times that of AGP (42 kDa). These results indicate that binding sites for AGP and anti-CCR5 mAb 2D7 might not be located in similar, but rather in spatially close, CCR5 regions. Alternatively, anti-CCR5 mAb 2D7 may bind, at the MDM surface, to multiple different CCR5 molecules characterized by different conformational states [24], while AGP may interact with some of these. Taken together, our results rule out a direct interaction of AGP with ECL2, and rather suggest that this glycoprotein may interact with a domain close to ECL2.

Finally, we have observed that, like V3Cs, native AGP (but not its heat-denatured counterpart) interacts specifically with electroblotted AGP-bound MDM membrane ligands of 33–36 kDa and 46 kDa; these proteins were characterized as CCR5 by their interactions with native MIP-1β, polyclonal antibodies specific for the CCR5 C-terminal domain and mAb 2D7 specific for ECL2. Interestingly, it has been shown in several different cell lines that a 36 kDa molecular mass form of CCR5 is expressed at a lower level than the mature form (46 kDa) [29]. The identity of this protein as a CCR5 isoform was confirmed by its precipitation by anti-CCR5 antibodies and by mass spectrometry [29]. In the present study, the fact that AGP-unbound membrane ligands did not interact with the polyclonal anti-CCR5 antibodies indicates that all of the MDM CCR5 molecules that are immunoreactive with these antibodies were bound to the AGP-coated beads. However, among the AGP-unbound proteins, we observed the presence of a 60 kDa protein which was immunoreactive with anti-CCR5 mAb 2D7. Taken together, these data demonstrate, at the molecular level, that AGP binds some, but not all, of the CCR5 molecules expressed at the MDM membrane. Since heat denaturation of AGP abolishes AGP binding to electroblotted CCR5, it can be concluded that the three-dimensional structure of AGP is involved in its binding to CCR5. These results rule out a role for AGP glycans, which are resistant

to heat denaturation. Indeed, preincubation of AGP with mannan or its pretreatment with N-Glycanase had no effect on the binding of AGP to electroblotted CCR5.

In conclusion, our data show that AGP binds specifically to macrophages via its glycans, most probably mannose residues interacting through low-affinity interactions with a macrophage membrane lectin or lectin-like receptor, and via CCR5. They suggest that the inhibitory effect of AGP on the R5 HIV-1 infection of human primary macrophages *in vitro*, observed previously in our laboratory [11], may be related to a specific interaction of AGP with a macrophage membrane lectin or lectin-like component and with a CCR5 domain close to ECL2. Our data also suggest that the three-dimensional structures of AGP and CCR5 are involved in AGP–CCR5 interactions. Thus AGP may contribute *in vivo* to a natural defence mechanism against the infection of macrophages by R5 HIV-1, which may depend on its serum level and on the viral load. Characterization of AGP domains involved in its binding to CCR5, and of the AGP and HIV-1 gp120 glycans involved in their interactions with macrophages, may lead to new therapeutic formulations able to prevent the entry of HIV-1 into these cells.

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