α_2 -Macroglobulin modulates the immunoregulatory function of the lipocalin placental protein 14

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Human placental protein 14 (PP14; also known as glycodelin and progesterone-associated endometrial protein) is an immunosuppressive protein of the lipocalin structural superfamily. Mechanisms regulating serum PP14's immunosuppressive activity remain to be elucidated. In the present study, an interaction between PP14 and a major serum protein carrier, α_2 -macroglobulin (α_2 M), was documented for the first time. Using native gel electrophoresis, we showed that PP14, as well as its alternative splice variant PP14.2, binds to both α_2 M and methylamine-activated (MA)- α_2 M. Cross-competition studies demonstrated that the variants compete for binding to α_2 M. PP14 bound to α_2 M and MA- α_2 M with K_d values of 167 ± 70 and 221 ± 56 nM

(means $\pm$ S.D.) respectively, as determined by surface plasmon resonance. Significantly, the addition of α_2 M or MA- α_2 M to a T-cell proliferation assay strongly potentiated the inhibitory capacity of PP14. On the basis of these findings, α_2 M emerges as the first serum protein that can physically associate with, and thereby regulate, PP14. Moreover, this represents the first documented interaction between the protein carrier α_2 M and a lipocalin protein.

Key words: glycodelin, immunosuppressive, pregnancy proteins, serum carrier proteins, serum transport.

INTRODUCTION

Placental protein 14 (PP14; also known as glycodelin or progesterone-associated endometrial protein) is a 28 kDa glycoprotein of the lipocalin structural superfamily. Lipocalins are a heterogeneous group of lipophile-binding proteins, including β -lactoglobulin and retinol-binding protein, that share defined primary sequence motifs and calyx-like β -barrel tertiary structures [1]. Glandular cells of secretory and decidualized endometrium are known to be a major source of PP14; this protein accumulates to high levels in amniotic fluid (AF). PP14 has also been recently detected in tumour cell lines derived from both breast and ovarian carcinomas [2,3]. In addition, mRNA encoding PP14 (162 residues), along with a shorter (129 residue) alternative splice variant, is expressed by human megakaryocytes and platelets [4].

The PP14 in female serum most probably originates from the endometrium [5]. During a typical menstrual cycle, PP14 reaches a serum concentration of approx. 50 ng/ml at 2 weeks after ovulation. During pregnancy, maternal serum PP14 concentrations peak at 0.6–2.2 μ g/ml at approx. 6–12 weeks after the last menstrual period [6]. By comparison, the concentration of PP14 in AF reaches 100 μ g/ml [6].

Although the precise physiological role of PP14 is not known, its immunosuppressive properties *in vitro* are of special interest [7]. In a limited set of early studies, PP14 was shown to block T-cell proliferation in mixed lymphocyte cultures [4] and to inhibit the secretion of interleukin 1 (IL-1) [8] and IL-2 [9] by phytohaemagglutinin-stimulated peripheral blood mononuclear cells. Our more extensive studies have recently documented a direct inhibitory effect of PP14 on T cells that is independent of

accessory cells [10], an immunoregulatory property that is uncommon and shared with transforming growth factor β (TGF- β) [11,12]. It is noteworthy that all of these immunological experiments were performed in the presence of fetal bovine serum; however, the potential contribution of serum components to PP14-mediated immunoinhibition in these assays was not assessed.

α_2 -Macroglobulin (α_2 M) is a major protein in serum with a large molecular mass of 725 kDa. It is secreted by many cell types, including those of the liver, adrenal glands and endometrium. The level of α_2 M in the serum varies in some disease states and increases in pregnancy [13]. α_2 M is not detectable in the AF of either normal or high-risk pregnancies [14].

First described as a protease inhibitor, α_2 M is now well known for its ability to bind and modulate the activity of growth factors and cytokines [15]. IL-2 [16], TGF- β_1 [17], platelet-derived growth factor (PDGF) [18], nerve growth factor (NGF) [19], and also other proteins, specifically bind to normal, protease-activated and methylamine-activated (MA)- α_2 M. α_2 M is the major serum carrier for some of these proteins and can bind to them non-covalently [19,20], covalently by means of thiol exchange [16], covalently during protease trapping [21] or by a combination of these mechanisms [22]. Beyond simply binding to proteins, α_2 M can also modulate their function, with pleiotropic effects that are context-dependent. For example, MA- α_2 M enhances the PDGF-mediated stimulation of rat lung fibroblasts [23], decreases the ability of TGF- β_2 to inhibit the growth of fetal bovine heart endothelial cells [24] and inhibits NGF-mediated signal transduction in neurons [25,26].

In the present study we link α_2 M to PP14 for the first time, characterizing the kinetics of their interaction and its functional

Abbreviations used: AF, amniotic fluid; α_2 M, α_2 -macroglobulin; IL, interleukin; MA, methylamine-activated; mAb, monoclonal antibody; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PP14, placental protein 14; rPP14, recombinant placental protein 14; SPR, surface plasmon resonance; TGF- β , transforming growth factor β ; RU, response units.

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consequences. PP14 is the first lipocalin of the diverse set of proteins known to bind to $\alpha_2\text{M}$. Viewed from the other side, $\alpha_2\text{M}$ becomes the first documented PP14-binding protein within serum; its PP14-modulatory effects are potentially significant in understanding PP14 function within different body compartments.

MATERIALS AND METHODS

Production and radioiodination of recombinant PP14 (rPP14) and rPP14 isoforms

The production of rPP14 and rPP14.2 is described elsewhere [27]. rPP14 was radiolabelled by lactoperoxidase-catalysed iodination. In brief, 10 μg of protein was mixed with 1 mCi of Na^{125}I (NEN Life Sciences, Boston, MA, U.S.A.) and 30 m-units of lactoperoxidase (Calbiochem, San Diego, CA, U.S.A.). The reaction was initiated by the addition of H_2O_2 (0.12 mM) and proceeded for 15 s before being stopped with 0.05% NaN_3 and 500 mM KI. Radiolabelled protein was separated from free iodine by gel-filtration chromatography over G-25 Sephadex (Pharmacia, Piscataway, NJ, U.S.A.). The column elution buffer consisted of PBS containing 0.05% NaN_3 and 0.1% BSA.

Immunoaffinity purification of human PP14

Sepharose-coupled rPP14 was used to affinity-purify polyclonal anti-PP14 antibody from rabbit immune serum [4]. The affinity-purified polyclonal antibody was coupled with CNBr-activated Sepharose 4B (Pharmacia) and the matrix was washed in accordance with the manufacturer's protocol. Pooled AF, buffered with 10 mM Tris/HCl, pH 7.5, was precleared with Sepharose CL-4B (Pharmacia) and then incubated with the anti-PP14 Sepharose for 24 h at 4°C. The matrix was washed with 500 mM NaCl/10 mM Tris/HCl (pH 7.5) and eluted with 100 mM glycine, pH 2.5, into 0.1 vol. of 1 M Tris/HCl, pH 8.0. Fractions containing the immunoaffinity-purified amniotic PP14 were concentrated and buffer-exchanged into PBS.

Preparation of $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$

Human $\alpha_2\text{M}$ was obtained from normal human plasma (Red Cross of Northern Ohio, Cleveland, OH, U.S.A.) containing 0.38% sodium citrate, by ion-exchange and gel-filtration chromatography [28]. The $\alpha_2\text{M}$ obtained was homogeneous as judged by native PAGE or SDS/PAGE [5% (w/v) gel]. Purified $\alpha_2\text{M}$ was quantified by an $A_{1\text{cm}}^{1\%}$ of 8.4 at 280 nm. MA- $\alpha_2\text{M}$ was prepared by incubating normal $\alpha_2\text{M}$ (2–3 mg/ml) in 0.02 M methylamine/0.05 M Tris/HCl (pH 8.1) as described [28].

Formation of PP14- $\alpha_2\text{M}$ complexes

Normal $\alpha_2\text{M}$ or MA- $\alpha_2\text{M}$ was incubated with ^{125}I -PP14 or ^{125}I -PP14.2 in PBS with 2.5 mg/ml BSA (Sigma Chemical Co., St Louis, MO, U.S.A.) and 2.5 mg/ml cytochrome *c*, in combination as carrier proteins, for 3 h at 37°C. In experiments in which the pH was altered, PBS was replaced with 100 mM phosphate buffers (pH 5.5–7.5) or 50 mM Tris buffers (pH 8.0–9.5). At the end of each incubation period, an aliquot containing 12 μg (16.5 pmol) $\alpha_2\text{M}$ and 0.8 ng (34 fmol) of ^{125}I -PP14 was mixed with 0.25 vol. of loading buffer [50% (w/v) sucrose/0.005% Bromophenol Blue] and applied to a 5% (w/v) native polyacrylamide gel, run in Tris/glycine buffer, pH 9.5. As described in the Results section, some samples were mixed with 0.2 vol. of $6\times$ SDS loading buffer [0.3% SDS/30% (v/v) glycerol/0.12 mg/ml Bromophenol Blue] and then applied to a 5% (w/v)

SDS gel. After electrophoresis the gels were fixed, dried and exposed to RX film (Fuji, Stamford, CT, U.S.A.).

Surface plasmon resonance (SPR)

All SPR experiments were done with a BIAcore 1000, sensor chips CM5, surfactant P20, *N*-hydroxysuccinimide, *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodi-imide and ethanolamine hydrochloride, all acquired from Biacore (Piscataway, NJ, U.S.A.). The running buffer in all SPR experiments was 10 mM Hepes (pH 7.4)/150 mM NaCl/1 mM EDTA/0.005% P-20 (HBS; Biacore). Immobilization was accomplished by the coupling of primary amines in accordance with the manufacturer's recommendations. Sufficient rPP14 was immobilized to obtain maximum response units (RU_{max}) of less than 200 RU with $\alpha_2\text{M}$ bound. All experiments were performed at a constant temperature of 25°C and a flow rate of 10 $\mu\text{l}/\text{min}$. During the association phase, 50–100 μl of the analyte $\alpha_2\text{M}$ solution was injected over a 5–10 min period. Dissociation proceeded with HBS as the running buffer for 5 min. Regeneration was achieved by washing with 1 M NaCl. The analyte solutions were checked for non-specific binding to both a flow cell that had been activated and blocked with the amine-coupling kit in the absence of PP14, as well as an unmodified CM5 flow cell. Non-specific binding to the PP14 immobilized on the flow cell was determined by using BSA as an analyte. With BSA, at a concentration 100-fold that of the $\alpha_2\text{M}$ used in these experiments, no binding to PP14 was observed. Sensorgrams were analysed by non-linear least-squares fitting with BIAevaluation 3.0 software (Biacore). Kinetic constants were determined by global analysis of the association and dissociation phases of at least three analyte concentrations. The data were fitted to a single-site binding model ($A+B=AB$). This model gave fits with χ^2 values of less than 10; all *T* values were greater than 1000. All kinetic constants given were obtained with $\alpha_2\text{M}$ concentrations between 100 nM and 1.5 μM and are represented as the average from experiments performed with different PP14-immobilized surfaces.

T-cell proliferation assay

Human peripheral blood mononuclear cells were obtained from normal laboratory volunteers and purified by Ficoll-Hypaque density-gradient centrifugation. The T cells were isolated by complement depletion with Lympho-Kwik T, as described previously [10]. The T cells were cultured in AIM-V (Gibco-BRL, Bethesda, MD, U.S.A.) serum-free medium and stimulated to proliferate by an anti-CD3 monoclonal antibody (OKT3, final concentration 2 $\mu\text{g}/\text{ml}$; prepared by Dr John Fayen, Case Western Reserve University, Cleveland, OH, U.S.A.) immobilized on Protein A-Sepharose (Sigma Chemical Co.); anti-CD28 mAb (9.3 clone; provided by Bristol-Myers Squibb) was added at a final concentration of 1 $\mu\text{g}/\text{ml}$. Human AF, as a source of PP14, and purified human $\alpha_2\text{M}$ or MA- $\alpha_2\text{M}$ were added as described in the figure legends. The T cells were left to proliferate for 72 h at 37°C under air/ CO_2 (19:1). To monitor proliferation, [^3H]thymidine (2.5 $\mu\text{Ci}/\text{ml}$) was added for the last 18 h. Cells were harvested on a Harvester 96 (Tomtec, Orange, CT, U.S.A.) and the ^3H incorporated was analysed on a 1205 BetaplateTM liquid-scintillation counter (Wallac, Gaithersburg, MD, U.S.A.). The results are expressed as percentage inhibition, which is calculated as $1 - [(\text{c.p.m. with AF})/(\text{c.p.m. without AF})]$ for each condition. Results are representative of at least three experiments done in triplicate.

RESULTS

Evidence for the formation of high-molecular-mass complexes between rPP14 and human plasma proteins

Gel-filtration chromatography was used to look for high-molecular-mass complexes formed between PP14 and human plasma proteins. Specifically, human plasma and radiolabelled rPP14 were incubated together at 37 °C for 3 h and the mixture was then applied to a Sepharose CL-6B column. Under these conditions, a large proportion of the radioactivity was associated with fractions of molecular masses greater than 500 kDa (Figure 1). This finding suggested that PP14 can bind to a high-molecular-mass protein within plasma.

Figure 1 Binding of ^{125}I -rPP14 to human plasma

^{125}I -rPP14 (4 ng) was tested alone (broken line) or mixed with (solid line) 50 μl human plasma in PBS and incubated for 2 h at 37 °C before being loaded on a 1 cm $\times$ 30 cm Sepharose CL-6B column.

Figure 2 Binding of ^{125}I -rPP14 to purified $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$ determined by native gel electrophoresis

(A) Radiolabelled rPP14 (0.8 ng) was incubated without (lane 1) or with 12 μg of purified human $\alpha_2\text{M}$ (lanes 2 and 3) or 12 μg of MA- $\alpha_2\text{M}$ (lanes 4 and 5) for 3 h at 37 °C; the mixture was then separated by 5% (w/v) PAGE. Unlabelled rPP14 was added as a competitive blocker (lanes 3 and 5) to demonstrate specificity. (B) Radiolabelled rPP14 (0.8 ng) was incubated with 5 μl of human plasma. (C) Radiolabelled human PP14 (4 ng) was incubated with 12 μg of $\alpha_2\text{M}$ (lane 1) or 12 μg of MA- $\alpha_2\text{M}$ (lane 2). The positions of free (asterisk) and bound (arrow) PP14 are indicated.

Binding of PP14 to purified $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$

Given the high molecular mass of $\alpha_2\text{M}$, as well as the paucity of other serum proteins with a similarly high molecular mass, $\alpha_2\text{M}$ was implicated at the outset as a potential PP14-binding protein. The documented ability of $\alpha_2\text{M}$ to interact with diverse growth factors and cytokines strengthened its candidacy. ^{125}I -rPP14 and $\alpha_2\text{M}$ purified from human plasma were incubated together at 37 °C for 3 h; the reaction mixture was then separated on a 5% (w/v) native polyacrylamide gel. As demonstrated in Figure 2(A), radiolabelled rPP14 bound to purified $\alpha_2\text{M}$ and the binding could be competitively inhibited with an excess of unlabelled rPP14.

Normal plasma $\alpha_2\text{M}$ must react with a protease or monoamine for it to be recognized by its cell-surface receptor, the low-density lipoprotein-receptor-related protein (LRP). Methylamine-activated $\alpha_2\text{M}$ (MA- $\alpha_2\text{M}$) is commonly used as a model for protease-reacted $\alpha_2\text{M}$ and it is known to differ from normal $\alpha_2\text{M}$ in its binding capabilities [29]. We determined whether MA- $\alpha_2\text{M}$ parallels $\alpha_2\text{M}$ in its capacity to bind to PP14. This did in fact occur (Figure 2A, lane 4), and the ^{125}I -rPP14 binding to MA- $\alpha_2\text{M}$ readily competed with unlabelled rPP14 (Figure 2A, lane 5).

We next examined whether the major PP14-binding protein in serum was indeed $\alpha_2\text{M}$. To this end, ^{125}I -rPP14 was combined with whole human plasma, in place of purified $\alpha_2\text{M}$, and complexes were detected by native gel electrophoresis. Under the same binding and electrophoresis conditions used for purified $\alpha_2\text{M}$, PP14 formed a single complex with human plasma that was detected by autoradiography (Figure 2B). This complex migrated with purified $\alpha_2\text{M}$ run on the same gel (results not shown), supporting the possibility that PP14 binds to $\alpha_2\text{M}$ in the context of whole plasma.

To show that PP14- $\alpha_2\text{M}$ association was not peculiar to recombinant periplasmically produced PP14, similar binding experiments were performed with radiolabelled immunoaffinity-purified PP14 from AF. As expected, this native amniotic, fully glycosylated ^{125}I -PP14 also bound to both $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$ (Figure 2C).

Binding of PP14.2 to purified $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$

Because platelet-derived PP14 might be of significance at sites of inflammation, we examined whether the platelet-specific isoform, PP14.2, also bound to $\alpha_2\text{M}$. As shown in Figure 3(A), PP14.2 mirrors PP14 in its ability to bind to $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$. In addition, the PP14.2 and PP14 splice variants competed with each other for binding to $\alpha_2\text{M}$ (Figure 3B), suggesting that the two isoforms might engage the same or neighbouring sites on $\alpha_2\text{M}$.

Disruption of PP14- $\alpha_2\text{M}$ complex formation by SDS

The nature of the interaction between PP14 and $\alpha_2\text{M}$ was further evaluated. Cytokines and growth factors bind $\alpha_2\text{M}$ either non-covalently [20] or covalently [16,21]. To distinguish between covalent and non-covalent binding, the stability of PP14- $\alpha_2\text{M}$ complexes was determined in the presence of SDS. PP14- $\alpha_2\text{M}$ complexes were formed and then separated by SDS/PAGE in the absence of a reducing agent. Incubation with SDS caused dissociation of the complexes, whereas control samples run under native conditions remained intact (results not shown), indicating that the PP14- $\alpha_2\text{M}$ association is non-covalent.

Dependence of complex formation on pH

To characterize the PP14- $\alpha_2\text{M}$ interaction further and gain insight into the physiological conditions that might allow the

Figure 3 Binding of ^{125}I -rPP14.2 to purified $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$, and competition by unlabelled rPP14

(A) Radiolabelled rPP14.2 was incubated without (lane 1) or with purified human $\alpha_2\text{M}$ (lanes 2 and 3) or MA- $\alpha_2\text{M}$ (lanes 4 and 5) for 3 h at 37 °C; the mixture was then separated by 5% (w/v) PAGE. Unlabelled rPP14 was added to demonstrate specificity (lanes 3 and 5). (B) Radiolabelled PP14.2 (1 ng) was incubated with human MA- $\alpha_2\text{M}$ and unlabelled PP14 (lane 2, 10 ng; lane 3, 50 ng; lane 4, 100 ng; lane 5, 1000 ng) or unlabelled PP14.2 (lane 7, 10 ng; lane 8, 50 ng; lane 9, 100 ng; lane 10, 1000 ng). The positions of free (asterisk) and bound (arrow) PP14.2 are indicated.

Figure 4 pH dependence of $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$ interactions

Radiolabelled PP14 was incubated with $\alpha_2\text{M}$ (A) or MA- $\alpha_2\text{M}$ (B) in buffers of various pH values (lanes 1, pH 5.5; lanes 2, pH 6.0; lanes 3, pH 6.8; lanes 4, pH 7.4; lanes 5, pH 8.5) and then separated by 5% (w/v) PAGE.

preferential association or dissociation of the PP14- $\alpha_2\text{M}$ complex, we examined the effect of pH on complex formation. The PP14- $\alpha_2\text{M}$ or PP14-MA- $\alpha_2\text{M}$ complexes were formed between pH 5.5 and 8.8. Under these experimental conditions, dissociation of the $\alpha_2\text{M}$ into half-molecules was not observed until the pH was less than 5.0 (results not shown). By native gel electrophoresis and autoradiography, PP14 was found to bind both $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$ most efficiently at pH 6.0–6.8 (Figure 4). This binding was significantly decreased at higher pH.

Kinetics of complex formation

SPR was used for quantitative characterization of the kinetics of the PP14- $\alpha_2\text{M}$ interaction. To our knowledge there have been no previous reports of the use of SPR for examining the kinetics of $\alpha_2\text{M}$ interactions, although in some instances affinity measurements have been made based on other methods. rPP14 was immobilized on a dextran sensor chip, and $\alpha_2\text{M}$ was used as the solution-phase analyte. To reduce the impact of mass transport limitations, PP14 was immobilized at low levels and the flow rate was kept at 10 $\mu\text{l}/\text{min}$. After the injection of various concen-

Figure 5 Analysis of interaction kinetics by surface plasmon resonance

rPP14 was immobilized on CM5 dextran sensor chip and $\alpha_2\text{M}$ (A) or MA- $\alpha_2\text{M}$ (B) was injected over the flow cell to monitor the formation of PP14- $\alpha_2\text{M}$ complexes. Sensorgrams (relative response in RU after background subtraction plotted against time) are presented for various concentrations of analyte. (A) $\alpha_2\text{M}$ concentrations from top to bottom: 1.92 μM , 972 nM and 486 nM. (B) MA- $\alpha_2\text{M}$ concentrations from top to bottom: 1.58 μM , 790 nM, 634 nM, 317 nM and 158 nM.

Table 1 Kinetic binding constants for PP14- $\alpha_2\text{M}$ interaction

Kinetic binding constants were calculated from SPR data as described in the Materials and methods section. The values shown are averages of values determined from at least three concentrations of analyte on two or more separately coupled sensor chips.

Globulin	$10^3 k_d (\text{s}^{-1})$	$k_a (\mu\text{M}^{-1} \text{s}^{-1})$	$K_d (\text{nM})$
$\alpha_2\text{M}$	1.59 ± 0.59	162 ± 42	167 ± 70
MA- $\alpha_2\text{M}$	1.18 ± 0.35	5290 ± 130	221 ± 56

trations of $\alpha_2\text{M}$ or MA- $\alpha_2\text{M}$ in the solution phase, the association and dissociation phases of the PP14- $\alpha_2\text{M}$ interaction were examined (Figure 5). Analyses included at least three analyte concentrations on two or more separately prepared sensor chips. By using global analysis, dissociation, association and equilibrium constants were obtained for both PP14- $\alpha_2\text{M}$ and PP14-MA- $\alpha_2\text{M}$ interactions (Table 1). The observed K_d values of interaction were 167 and 221 nM for $\alpha_2\text{M}$ and MA- $\alpha_2\text{M}$ respectively. In other experiments, qualitatively similar binding was observed when the components were reversed, that is, $\alpha_2\text{M}$ was immobilized on the sensor chip and PP14 was added in the solution phase (results not shown). However, this experimental permutation could not be fully evaluated owing to the difficulty in finding a regeneration condition under which PP14 would be released from the $\alpha_2\text{M}$ yet would leave the tetrameric immobilized $\alpha_2\text{M}$ intact.

Effect of $\alpha_2\text{M}$ on PP14 function

To investigate the functional significance of PP14- $\alpha_2\text{M}$ binding, an *in vitro* assay monitoring the influence of PP14 on T-cell

activation was employed. Purified human T-cells were stimulated to proliferate by using immobilized anti-CD3 mAb and anti-CD28 mAb, in the presence or absence of AF in serum-free medium. We have shown in a previous study that the inhibitory effect of AF in this assay is due solely to PP14 [10]. It is noteworthy that α_2 M is absent from AF [14]. α_2 M or MA- α_2 M added alone to the T cells caused a small increase in proliferation (2.6% and 12.0% respectively). In the presence of AF alone, there was no inhibition of T-cell proliferation under these serum-free conditions. However, when both α_2 M (0.2 mg/ml) and AF were added in combination, there was a $32.7 \pm 3.3\%$ (mean $\pm$ S.D.) inhibition of proliferation. When MA- α_2 M was added in place of native α_2 M, the level of inhibition increased to $62.6 \pm 7.1\%$. Similar results were obtained when 10% (v/v) total serum was used instead of purified α_2 M or MA- α_2 M (results not shown). Therefore the addition of native α_2 M or activated α_2 M greatly augmented the ability of amniotic PP14 to inhibit T-cell proliferation in proliferation assays performed under serum-free conditions.

DISCUSSION

The present study documents the binding of PP14, a lipocalin with direct T-cell-suppressive properties, to α_2 M, a major carrier protein in serum. This finding adds PP14 to the collection of proteins with known α_2 M-binding capacities. The PP14- α_2 M interaction is specific, non-covalent and pH dependent. Furthermore, the physical interaction is functionally significant in that α_2 M, as well as its activated form, MA- α_2 M, can potentiate the immunoinhibitory effect of PP14 on T-cell activation.

PP14 is the first lipocalin with documented α_2 M-binding capacity. Our kinetic analysis of the binding of PP14 to α_2 M and MA- α_2 M by SPR determined K_d values of approx. 100 nM. Of the numerous reports of proteins binding to α_2 M, few have attempted to describe the α_2 M association quantitatively. Where reported, affinities for binding to α_2 M have been in the range 1 μ M to 10 nM; these values were determined by either an equilibrium binding method [19,30] or a gel electrophoresis method dependent on bis(sulphosuccinimidyl) suberate-mediated cross-linking [31]. With the use of yet another method, namely SPR, the interaction of PP14 with α_2 M is here shown to be comparable to that calculated for other α_2 M-associating proteins. It would be of interest to examine other lipocalins for their capacity to bind α_2 M, in addition to applying SPR to other classes of α_2 M-associated protein.

It is interesting to note that native and monoamine-activated α_2 M have similar K_d values of interaction with PP14. Most of the well-characterized α_2 M-interacting proteins bind to the native form with a lower affinity than to MA- α_2 M. This has been shown to be true for TGF- β_1 , NGF, PDGF, basic fibroblast growth factor, tumour necrosis factor α and IL-8 [30–32]. However, for TGF- β_2 [31], and now for PP14 (Table 1), the affinities for α_2 M and MA- α_2 M are comparable. Therefore the monoamine reaction with cyclic thioester bonds in α_2 M does not necessarily increase the affinity of α_2 M for all cytokines, as had been suggested.

Although α_2 M and MA- α_2 M bind equally well to PP14, MA- α_2 M more potently induces the immunoinhibitory activity of PP14. PDGF and TGF- β_1 activities are also upregulated preferentially by MA- α_2 M in comparison with α_2 M [33,34]. For these growth factors, the mechanism responsible for the augmentation of activity by MA- α_2 M was postulated to involve interaction with α_2 M receptors. The activation of α_2 M to form MA- α_2 M causes a conformational change that alters its ability to bind α_2 M receptors. Both the low-density lipoprotein-receptor-related

protein and the α_2 M signalling receptor bind only the activated form of α_2 M [35]. However, because neither of these receptors is found on T cells, the enhanced potentiating activity of MA- α_2 M for PP14 is not likely to stem from receptor-related factors.

There are several other hypotheses that could account for the effect of α_2 M on PP14 activity. First, α_2 M might act as a scaffold for the formation of higher-order complexes of PP14, which in turn could enable the cross-linking of cell-surface PP14 receptors. Secondly, the PP14 regulatory activity of α_2 M might reflect its well-described protease inhibitor activity, with α_2 M preventing PP14 degradation in T-cell proliferation assays. Thirdly, it is possible that the interaction between α_2 M and PP14 leads to a conformational change that activates PP14.

Because the interaction between PP14 and α_2 M is non-covalent, unloading of PP14 from α_2 M might occur readily. Therefore α_2 M could have a role in the transport of PP14 to functional sites. Furthermore, the finding that PP14- α_2 M binding can be regulated by pH might have physiological relevance. The pH-dependent interaction between α_2 M and PP14 might influence the anti-inflammatory activity of PP14 in anatomical locations with low pH, such as sites of inflammation [36].

The present study has identified α_2 M as the first PP14-binding protein, as well as the first protein capable of modulating the function of PP14. Previous assays of the immunoregulatory activity of PP14 by us [4,10] and others [7,8,37] were performed in the presence of serum. By using serum-free conditions, the present study has unmasked the dependence of PP14 on serum-supplied factors.

α_2 M is absent from AF [14], the anatomical compartment with the highest known concentration of PP14. Taken together with the finding of the dependence of PP14 on α_2 M, the intriguing possibility emerges that the latent immunoinhibitory potential of PP14 might become manifest only once PP14 has exited from the amniotic sac and entered the systemic circulation. This constitutes a unique system in which to explore differential immunoregulatory functions for a given protein in different body compartments.

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