Synthesis of a Cys949Tyr α_2 -macroglobulin thiol ester mutant: co-transfection with wild-type α_2 -macroglobulin in an episomal expression system

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A full-length α_2 -macroglobulin (α_2 M) cDNA was cloned into the episomal expression vectors pREP7 and pMEP4. Electroporation of the cell lines WI-L2-729HF2, U-937, K-562 and an Epstein–Barr virus-transformed cell line resulted in stable transfectants only with K-562 cells. Stable expression was obtained exclusively with pMEP4- α_2 M and was driven from the inducible human metallothionein II_A promoter. Expression of the wild-type α_2 M cDNA resulted in a recombinant protein ($r\alpha_2$ M) that could not be distinguished from plasma α_2 M (p- α_2 M): the transfected K-562 cells secreted tetrameric α_2 M with intact internal thiol esters, a functional bait domain and a latent receptor-binding domain. $r\alpha_2$ M inhibited trypsin and elastase from cleaving a high-molecular-mass substrate. When the Cys-949 involved in the formation of the internal thiol ester was mutated to tyrosine (C949Y- $r\alpha_2$ M), a tetrameric α_2 M was secreted, with the electrophoretic mobility of methylamine-treated p- α_2 M (p- α_2 M/MA)

and with a functional receptor-binding domain. The C949Y- $r\alpha_2$ M did not possess proteinase-inhibiting capacity. Heterozygosity was mimicked by co-transfecting the K-562 cells with wild-type and mutant expression vectors. In this case, $r\alpha_2$ M was secreted with zero, one, two, three or four internal thiol esters. A comparison of the interaction of interleukin 1 β and basic fibroblast growth factor with native p- α_2 M, p- α_2 M/MA and the mutant C949Y- $r\alpha_2$ M revealed that when assayed under non-denaturing conditions, no binding occurred to ‘slow’ p- α_2 M whereas quantitatively similar binding was observed to ‘fast’ p- α_2 M/MA and C949Y- $r\alpha_2$ M. Covalent binding, however, was essentially limited to p- α_2 M/MA, suggesting the involvement of Cys-949 in the process. Covalent binding of insulin, on the contrary, was only observed when it was present during hydrolysis of the internal thiol esters of p- α_2 M by trypsin treatment, and thus involves the activated Glx residue.

INTRODUCTION

Human α_2 -macroglobulin (α_2 M) is a tetrameric glycoprotein with a molecular mass of 720 kDa. The α_2 M monomer contains a unique internal thiol ester (ITE) formed between the side chains of Cys-949 and Gln-952 (reviewed in ref. [1]). In humans it shares this property with complement components C3 and C4. The ITE plays a central role in the function of α_2 M as a protease inhibitor. The cleavage of a bait domain by a protease triggers the hydrolysis of the thiol ester. On the one hand this results in a conformational change whereby the protease is trapped within the α_2 M molecule [2]. Secondly, the activated Glx residue generated in the process is able to bind covalently the protease or other polypeptides by the formation of an amide bond with α -amino groups of peptides or ϵ -amino groups of lysine residues [3,4]. The conformational change also induces the expression of a receptor-binding domain which then mediates binding of the α_2 M–protease complexes to the cellular α_2 M low-density-lipoprotein receptor-related protein (LRP) receptor and their clearance through the process of receptor-mediated endocytosis [5].

Poller et al. [6] described the detection of a Cys-959 to Tyr mutation in the α_2 M gene of a patient with chronic obstructive lung disease. The patient was heterozygous for this mutation, which must result in the absence of an ITE in the mutant α_2 M monomers. The serum α_2 M level of the patient was within the normal range. This raises questions about the effect of this mutation on the correct secretion and functionality of the mutant

α_2 M and on the effect of the intracellular presence of both mutant and wild-type monomers on the assembly of the α_2 M tetramer.

Many cytokines and growth factors have been reported to bind to plasma α_2 M (p- α_2 M). These cytokines bind α_2 M according to different mechanisms: (i) non-covalent binding to native and inactivated α_2 M; (ii) non-covalent entrapment of the cytokine during the conformational change associated with the inactivation of α_2 M; (iii) covalent binding through a thiol–disulphide exchange mechanism; (iv) covalent binding via the interaction of nucleophilic groups of the cytokine with the reactive glutamine residue that becomes available after thiol ester breakage (reviewed in ref. [7]). Although many growth factors bind in a non-covalent manner to α_2 M, some, such as interleukin 1 β (IL-1 β), platelet-derived growth factor, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor, bind covalently [8–15], presumably via a thiol–disulphide exchange mechanism. A determining factor for this is the presence of free thiol groups on the cytokine. IL-1 β and bFGF for example have respectively two and four free thiols [16–20]. To study the C949Y mutation *in vitro*, an episomal expression system was developed for recombinant α_2 M ($r\alpha_2$ M). This system also allowed us to mimic the heterozygous situation by co-transfecting wild-type α_2 M (WT- $r\alpha_2$ M) and mutant C949Y- $r\alpha_2$ M cDNA into K-562 cells. Moreover, as the C949Y- $r\alpha_2$ M lacks the cysteine residue that takes part in ITE formation, it was used to investigate the role of this residue in the thiol–disulphide exchange between α_2 M and cytokines.

Abbreviations used: α_2 M, α_2 -macroglobulin; p- α_2 M, plasma α_2 -macroglobulin; $r\alpha_2$ M, recombinant α_2 -macroglobulin; ITE, internal thiol ester; EBV, Epstein–Barr virus; DMEM/F12, Dulbecco’s modified Eagle’s medium/Ham’s F12 medium; WT, wild-type; IL-1 β , interleukin 1 β ; bFGF, basic fibroblast growth factor; C949Y- $r\alpha_2$ M, $r\alpha_2$ M containing a Cys-949 to Tyr-949 mutation; p- α_2 M/MA, p- α_2 M treated with methylamine; LRP, low-density-lipoprotein receptor-related protein.

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EXPERIMENTAL

Episomal expression constructs for α_2 M

A full-length α_2 M cDNA was isolated from a liver cDNA library constructed by us and sequenced by standard methods. The α_2 M cDNA was cloned into the episomal expression vectors pREP7 and pMEP4 (Invitrogen) [21]. The pREP7 vector carries the rous sarcoma virus long terminal repeat promoter and a simian virus 40-derived polyadenylation signal. The pMEP4 vector contains the same polyadenylation signal but expression is driven by the inducible human metallothionein II_A promoter. Site-directed mutagenesis was performed using the 'Altered sites *in vitro* mutagenesis system' (Promega) as prescribed. The multiple cloning site of the pAlter1 vector was extended by the addition of a 31 bp sequence containing *Bst*XI, *Stu*I, *Mlu*I, *Xho*I and *Nsi*I restriction sites between its *Eco*RI and *Kpn*I sites (pAlter1B-N). A 736 bp *Stu*I-*Xho*I cassette containing the thiol ester site was subcloned in pAlter1B-N. The Cys-949 to Tyr mutation was introduced by means of a 21 bp oligonucleotide (5'-GCC CTA TGG CTA TGG AGA GCA-3'). All constructs were checked by sequencing.

Tissue culture and production of α_2 M

Human diploid fibroblast cells were cultured as described [22]. K-562, U-937, WI-L2-729HF2 cells and an Epstein-Barr virus (EBV)-transformed lymphoblast cell line were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) supplemented with 10% fetal calf serum. Transfections were performed by electroporation of 5×10^6 cells with 30 μ g of supercoiled expression vector (or $2 \times 15 \mu$ g in co-transfections) at 270 V and 960 μ F (Gene Pulser; Bio-Rad) in medium with 10% serum. The cells were kept on ice for 5 min before electroporation and at room temperature for 5 min after electroporation. Selection was performed using 200 μ g/ml hygromycin, resulting in stable transfectants after 2 weeks.

For the production of α_2 M, transfected cells were washed once in DMEM/F12, seeded at 4×10^5 cells/ml in DMEM/F12 supplemented with BSA (1 mg/ml), insulin (7 μ g/ml), transferrin (5 μ g/ml) and sodium selenite (7 ng/ml) and cultured for 24 h. The cells were then resuspended at 8×10^5 cells/ml in fresh DMEM/F12 medium supplemented with BSA (1 mg/ml), leupeptin (2 μ g/ml), aprotinin (2 μ g/ml), pepstatin (1 μ g/ml), bestatin (2 μ g/ml) and, when the pMEP4 vector was used, 3 μ M CdCl₂. The medium was harvested after 17–20 h by centrifugation at 300 g, adjusted to pH 6.6 with 0.5 M sodium phosphate, pH 6.0, and centrifuged again.

Purification of wild-type and mutant α_2 M

The culture medium was loaded on a Zn²⁺-saturated chelating Sepharose Fast Flow (Pharmacia) column (8 cm $\times$ 2 cm), equilibrated with 50 mM sodium phosphate, pH 6.5, containing 350 mM NaCl at 10 ml/min. The column was washed with the equilibration buffer and most of the contaminating proteins were eluted by applying a linear gradient of 20–38 mM imidazole in 40 mM Bistris, pH 6.8, over 5 min at 3 ml/min and washing the column with 38 mM imidazole until the absorbance of the eluate at 280 nm reached 0.1. The α_2 M was eluted with 50 mM EDTA/40 mM Bistris, pH 6.8. The fractions containing α_2 M were pooled, diluted 20-fold in 20 mM imidazole, pH 6.8, and applied to a QMA anion exchanger (QMA-MemSep 1000; Millipore). α_2 M was eluted by applying a linear gradient from 0 to 400 mM NaCl in 20 mM imidazole, pH 6.8, over 15 min at 4 ml/min. Gel filtration was performed by HPLC (Bio-Sil SEC-400; 300 mm $\times$ 7.8 mm; Bio-Rad) in 50 mM sodium phosphate,

pH 7.5/100 mM NaCl. The yield of α_2 M was determined using a specific ELISA [23] or by rocket immunoelectrophoresis.

For *in vivo* metabolic labelling, 1×10^6 cells were washed once in methionine-free DMEM (Gibco-BRL) and resuspended in the same medium (1.7 ml) containing 3 μ M CdCl₂ and the appropriate amount of [³⁵S]methionine. The supernatant was harvested after 15 h of incubation at 37 °C and purified on a Zn²⁺-saturated chelating column (200 μ l bed volume).

Characterization of α_2 M

Non-denaturing electrophoresis in 5% polyacrylamide slab gels was as described by Van Leuven et al. [24]. Gels (16 cm $\times$ 18 cm $\times$ 0.15 cm) were run at 4 °C. SDS/PAGE was performed on 7.5% homogeneous and 8–16% linear gradient (Novex) slab gels by the procedure of Laemmli [25]. All samples were reduced and heated for 15 min at 95 °C.

Trypsin treatment and methylamine treatment were performed as described previously [24]. α_2 M was iodinated and used for receptor competition studies as described by Van Leuven et al. [5]. All experiments were performed in triplicate.

TosPheCH₂Cl-treated trypsin was obtained from Sigma and porcine pancreatic elastase from Calbiochem.

Inhibition of proteolysis of resorufin-labelled casein

Resorufin-labelled casein was obtained from Boehringer. WT- α_2 M and C949Y- α_2 M (7 μ g) were incubated with or without 0.2 μ g of trypsin or elastase in 10 μ l buffered with Tris/HCl, pH 8.0 (50 mM final concn.). After 15 min at 37 °C 35 μ l of water, 22.5 μ l of 0.2 M Tris/HCl/0.02 M CaCl₂, pH 7.8, and 22.5 μ l of resorufin-labelled casein [0.4% (w/v) in redistilled water] were added. The mixture was incubated for 1 h at 37 °C. The reaction was stopped by the addition of trichloroacetic acid [5% (w/v) final concn.). The precipitate was pelleted by centrifugation. The supernatant (200 μ l) was then mixed with 300 μ l of 0.05 M Tris/HCl, pH 8.8, and *A*₅₇₄ measured against a blank without α_2 M and protease. The mean absorbance values of duplicate assays are given.

Interaction of p- α_2 M and α_2 M with cytokines

¹²⁵I-labelled recombinant human IL-1 β (76 μ Ci/ μ g), ¹²⁵I-labelled recombinant human bFGF (110 μ Ci/ μ g) and ¹²⁵I-labelled porcine insulin (85 μ Ci/ μ g) were purchased from NEN-Dupont. Glycine was removed from the ¹²⁵I-insulin preparation by size-exclusion chromatography over a G-10 column (Pharmacia) equilibrated in 50 mM Hepes/350 mM NaCl/0.1% BSA, pH 7.0. Methylamine-treated α_2 M (p- α_2 M/MA) was dialysed against 50 mM Hepes/100 mM NaCl, pH 7.0, to remove excess methylamine. p- α_2 M or α_2 M (1 μ M in each case) was incubated with 20 nM ¹²⁵I-cytokine in 14 μ l of buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.5, 0.15% BSA) for 1 h at 37 °C, or for 15 min at room temperature when trypsin (1:1 molar ratio of α_2 M to trypsin) was added. Free thiol groups were then blocked by treatment with iodoacetamide (50 mM final concn.) for 15 min at room temperature. Half of the reaction mixture was then treated with SDS [1% (w/v) final concn.]. Analysis of the samples by PAGE was as described above. Densitometric analysis was performed with a PhosphorImager (Molecular Dynamics).

RESULTS

Episomal expression system of α_2 M

A full-length α_2 M cDNA was cloned into the episomal expression

Figure 1 Characterization of WT- α_2 M (a,b) and C949Y- α_2 M (c,d)

(a) and (c) Native Page (5% gel); (b) and (d) SDS/PAGE (7.5% gel). p- α_2 M (P), WT- α_2 M (W) and C949Y- α_2 M (M) were untreated (—) or treated with methylamine (MA), trypsin (TR) or 1 % SDS (SDS).

vector pREP7 and transfected into four different cell lines without constitutional α_2 M expression. These were an EBV-transformed lymphoblast cell line, the lymphoblast cell line WI-L2-729HF2, the human lymphoma U-937 cell line and the erythroleukaemia cell line K-562. Stable transfectants were obtained only with the WI-L2-729HF2 and the K-562 cell lines. No α_2 M secretion by WI-L2-729HF2 cells was detected although episomes could be detected by Southern blotting. The transfected K-562 cells secreted significant amounts of α_2 M in the medium (up to 4 mg/l after 5 days) but this level of expression was not stable, with a 4-fold decrease in expression after 2 weeks in culture and no α_2 M secretion after 8 weeks. When the α_2 M cDNA was cloned into pMEP4, featuring the inducible human metallothionein II_A promoter, secretion of α_2 M could be induced in long-term cultures of transfected K-562 cells. This suggests that the rapid loss of α_2 M secretion in long-term cultures transfected with pREP7- α_2 M is the result of a strong selection against α_2 M-synthesizing cells. Cadmium was chosen as inducer rather than zinc or copper because the latter might interfere with the activity of α_2 M or give rise to dimerization [26] at the concentration needed for induction.

Recombinant wild-type α_2 M (WT- α_2 M)

WT- α_2 M was purified as described in the Experimental section and compared with p- α_2 M. WT- α_2 M could not be distinguished from p- α_2 M by PAGE under either native or denaturing conditions. WT- α_2 M migrated with the same mobility as native p- α_2 M ('slow' α_2 M [27]) in non-denaturing 5 % polyacrylamide

Table 1 Inhibition of trypsin and elastase by WT- and C949Y- α_2 m

Resorufin-labelled casein was incubated for 1 h with trypsin and elastase in the presence or absence of WT- α_2 M and C949Y- α_2 M as described in the Experimental section. The absorbance of trichloroacetate-soluble label was then measured at 574 nm.

	Enzyme activity ($\Delta A_{574}/h$ per 100 μ mol of protease)		
	Trypsin	Elastase	No protease
WT- α_2 M	0.134	0.204	0
C949Y- α_2 M	0.544	0.473	0
No α_2 M	0.524	0.475	0

gels (Figure 1a). After treatment of WT- α_2 M with methylamine or trypsin, its mobility in these gels was increased similarly to the mobility of methylamine- or trypsin-treated p- α_2 M ('fast' α_2 M). Treatment of WT- α_2 M with 1 % SDS before PAGE generated fragments with a mobility identical with the 360 kDa dimers generated from p- α_2 M under identical circumstances.

SDS/PAGE of heat-treated WT- α_2 M under reducing circumstances generated 180 kDa monomers and its 120 and 60 kDa heat fragments (Figure 1b). Treatment of WT- α_2 M with methylamine before heat treatment prevented the generation of 120 and 60 kDa heat fragments. After trypsin treatment, the 180 kDa bands disappeared and 90 kDa fragments were generated. Taken together, these results demonstrate secretion

Figure 2 Binding of the monoclonal antibody F2B2

Native PAGE (5% gel) of p- α_2 M, WT- α_2 M and C949Y- α_2 M (MUT) treated with trypsin and incubated with F2B2 (F2B2) as indicated was carried out. C, p- α_2 M stored at 4 °C for several months. The gel was stained with silver.

of WT- α_2 M with salient structural and functional characteristics of p- α_2 M. The WT- α_2 M monomers form disulphide-bridged dimers which assemble non-covalently into tetramers. Heat fragmentation, and its absence after methylamine treatment, suggests the presence of an intact ITE. The bait region is cleaved by trypsin and a conformational mechanism is active, as shown by the conversion of 'slow' WT- α_2 M into 'fast' WT- α_2 M on treatment with methylamine or trypsin.

C949Y- α_2 M

The episomal expression system was then used to study the C949Y mutation observed previously *in vivo* [6]. A C949Y mutation was introduced into the α_2 M cDNA *in vitro* and the mutant cDNA was cloned into pMEP4. K-562 cells were transfected with the C949Y-pMEP4 and recombinant mutant protein (C949Y- α_2 M) was purified similarly to WT- α_2 M and analysed as described above. The yield of C949Y- α_2 M was identical with that of WT- α_2 M. The untreated mutant protein migrated as 'fast' α_2 M on non-denaturing PAGE (Figure 1c). Treatment with trypsin or methylamine did not affect this behaviour. Incubation of the mutant in 1% SDS generated 360 kDa dimers. When the protein was heated for 15 min at 95 °C, no heat fragments were observed on reducing SDS/PAGE, indicating the absence of thiol esters (Figure 1d). Also, a trypsin treatment that converted the 180 kDa monomers into 90 kDa fragments for p- α_2 M converted only some of the C949Y monomers, indicating that the C949Y- α_2 M bait region has a lowered susceptibility to proteinases. To investigate whether the mutant retained any protease-inhibiting capacity, trypsin and elastase were incubated with resorufin-labelled casein in the presence and absence of WT- α_2 M and C949Y- α_2 M. Whereas WT- α_2 M protected this high-molecular-mass (23.6 kDa) substrate significantly from proteolytic degradation by trypsin and elastase (Table 1), C949Y- α_2 M did not show any inhibiting activity towards either proteinase. The residual proteolytic activity observed with WT- α_2 M is very similar to that observed with p- α_2 M [28].

The hydrolysis of the thiol ester in p- α_2 M triggers both the conformational change of the molecules and the exposure of a receptor-binding domain, mediating recognition of α_2 M by the α_2 M/LRP receptor. The receptor-binding domain is also recognized by the monoclonal antibody F2B2 [29]. Incubation of C949Y- α_2 M, trypsin-treated WT- α_2 M and p- α_2 M with F2B2 resulted in an identical decrease in mobility on non-denaturing PAGE, demonstrating expression of the receptor-binding domain in the three molecules (Figure 2).

Receptor binding of the two recombinant α_2 Ms was further analysed by investigating their ability to compete with labelled

Table 2 Inhibition of binding of 125 I-p- α_2 M/trypsin to fibroblast cell layers by p- α_2 M, WT- α_2 M and C949Y- α_2 M

Binding of 125 I-labelled p- α_2 M/trypsin to normal human fibroblasts was measured as described in the Experimental section. The cell layers were incubated for 4 h at 4 °C with medium containing 1 μ g/ml 125 I-p- α_2 M/trypsin (specific radioactivity 1750 c.p.m./ng) and 1% BSA. For the competition experiments, 1 μ g/ml p- α_2 M or WT- α_2 M (both treated with methylamine) or C949Y- α_2 M was added to the incubation medium. The mean of triplicate assays is given.

Additions (1 μ g/ml)	Inhibition of specific binding (%)
None	0
p- α_2 M/MA	75
WT- α_2 M/MA	78
C949Y- α_2 M	71

Figure 3 Characterization of metabolically labelled WT/C949Y- α_2 M

An autoradiograph is shown of a native 5% polyacrylamide gel of labelled WT/C949Y- α_2 M (CO) and WT- α_2 M (WT) treated with trypsin followed by incubation with F2B2 as indicated.

trypsin-treated α_2 M for binding to the α_2 M/LRP receptor. Normal human skin fibroblasts were incubated with 1 μ g/ml 125 I- α_2 M and unlabelled α_2 M (1 and 100 μ g/ml). The p- α_2 M/MA and WT- α_2 M/MA as well as the C949Y- α_2 M competed to the same extent with the labelled compound for specific binding to the receptor (Table 2).

Co-transfection of WT- α_2 M and C949Y- α_2 M

To investigate the association of α_2 M monomers generated by different alleles as described by Poller et al. [6] in a patient heterozygous for the C949Y mutation, co-transfection experiments were performed. The episomal expression system allowed us to mimic a situation where oligomeric proteins are built up as expression products of two (or more) different alleles or genomic sequences. K-562 cells were co-transfected with identical amounts of pMEP4-WT and pMEP4-C949Y. As soon as outgrowth of transfectants was seen (9 days after transfection), α_2 M was metabolically labelled and analysed. The labelled α_2 M derived from the co-transfection (WT/C949Y- α_2 M) migrated as a five-band pattern on a non-denaturing 5% polyacrylamide gel (Figure 3). The upper and lower band migrated with the same mobility as the 'slow' WT- α_2 M and the trypsin 'fast' WT- α_2 M respectively. This migration pattern is identical with that described previously for samples kept in the

Figure 4 Native PAGE of metabolically labelled WT-C949Y- α_2 M and WT- α_2 M

Purified WT/C949Y- α_2 M (CO) obtained after a 7-week culture period and WT- α_2 M (WT) were treated with trypsin followed by incubation with F2B2 as indicated.

refrigerator for longer times [27,30,31] (Figure 2, lane C). Trypsin treatment converts the five bands into one of 'fast' mobility (Figure 3). Incubation of WT/C949Y- α_2 M with F2B2 decreases the mobility of the four lower bands, indicating that these forms have at least one exposed receptor-binding domain. SDS/PAGE analysis revealed no 90 kDa fragments, which are the hallmark of proteolytic degradation (results not shown). Long-term culture (7 weeks) of co-transfected K-562 cells resulted in segregation of the mutant and wild-type episomes. Only 'slow' and 'fast' bands were seen on native PAGE (Figure 4).

Interaction of cytokines with p- α_2 M and C949Y- α_2 M

In order to study the involvement of Cys-949 in the binding of cytokines and growth factors, the interaction of IL-1 β , bFGF and insulin with C949Y- α_2 M was compared with the binding to p- α_2 M. IL-1 β and bFGF were chosen as they were previously reported to bind p- α_2 M via a thiol-disulphide exchange mechanism [9,14]. Insulin, covalently incorporated in α_2 M only during the proteinase-initiated conformational change through a transamidation [32], served as a control.

WT- α_2 M and C949Y- α_2 M were incubated with 125 I-labelled IL-1 β , bFGF or insulin as described in Experimental section. The products were then analysed under native and denaturing conditions.

The interaction between 125 I-IL-1 β and p- α_2 M, p- α_2 M/MA or C949Y- α_2 M was first studied by native PAGE (5% gel). Under these conditions a strong interaction was observed with p- α_2 M/MA, a very similar signal was observed with C949Y- α_2 M and there was only a very weak interaction with p- α_2 M. Treatment of the samples with SDS at room temperature, and separation by native PAGE, did not affect the signal observed with p- α_2 M/MA, whereas the majority of label was released from the C949Y- α_2 M dimers, which now showed a signal similar to that observed with p- α_2 M (Figure 5a). The association of 125 I label with p- α_2 M/MA was resistant to boiling and separation in a Laemmli gel system under non-reducing circumstances, demonstrating the covalent binding of 125 I-IL-1 β to the p- α_2 M/MA monomer (Figure 5b). This binding completely disappeared after treatment with 2-mercaptoethanol before electrophoresis, demonstrating that the binding occurs via a disulphide bond. The commercial preparation of 125 I-IL-1 β contained some labelled high-molecular-mass species, which precluded an exact estimation of IL-1 β binding to p- α_2 M or C949Y- α_2 M resistant to SDS/PAGE (Figure 5b). It is however, clear that this binding cannot exceed 10% of that to p- α_2 M/MA. Taken together, these results show that strong binding of 125 I-IL-1 β occurs essentially only to 'fast' α_2 M (p-

α_2 M/MA and C949Y- α_2 M). With p- α_2 M/MA the majority of the binding is covalent and occurs through a disulphide bond, whereas at least 80% of the binding to C949Y- α_2 M appears to be non-covalent and is not resistant to boiling in the presence of SDS.

Analysis of the interaction of 125 I-bFGF with α_2 M by native PAGE reveals binding to p- α_2 M, p- α_2 M/MA and C949Y- α_2 M (Figure 5c). A careful analysis of the mobility of the label in the first lane with p- α_2 M, however, shows that this does not correspond to 'slow' α_2 M (S), but to the next intermediate p- α_2 M band (S-1), generated on storage of the p- α_2 M in the cold. Treatment with SDS at room temperature (Figure 5c) or 100 °C (Figure 5d) substantially reduced the binding to C949Y- α_2 M but not to p- α_2 M or p- α_2 M/MA. The weaker signal observed with p- α_2 M throughout the experiment can be explained by the presence of intermediate p- α_2 M (S-1) at a much lower concentration (Figure 5c). The 125 I-bFGF- α_2 M complexes migrated as a smear on native PAGE after treatment with SDS at room temperature. An anomalous migration of these complexes on non-denaturing PAGE was also observed by Dennis et al. [14]. The covalent binding of 125 I-bFGF was completely abolished by treatment with 2-mercaptoethanol (Figure 5d). Taken together, these results show specific binding of 125 I-bFGF to 'fast' and 'intermediate' α_2 M, but not to 'slow' α_2 M. In the presence of Cys-949, approx. 50% of the binding is covalent, via a disulphide link. In the absence of Cys-949, the majority of the binding is non-covalent, although some covalent binding does occur.

A different picture was obtained with 125 I-insulin. When it was incubated with p- α_2 M, p- α_2 M/MA and C949Y- α_2 M and trypsin was added to the mixtures, strong binding was obtained only with p- α_2 M (Figures 5e and 5f). The binding to p- α_2 M was resistant to boiling in SDS, and reduction with 2-mercaptoethanol decreased the signal intensity by approx. 50%. Binding to p- α_2 M/MA and C949Y- α_2 M was less than 10% of that to p- α_2 M (Figure 5e) and similar to that observed in the absence of trypsin (not shown). This binding was non-covalent as it was not resistant to treatment with SDS and 2-mercaptoethanol (Figure 5f). Insulin thus became covalently bound to the reactive glutamate residue generated after thiol ester breakage by trypsin treatment of p- α_2 M as previously reported by Chu et al. [32]. The decreased signal seen on SDS/PAGE after reduction is due to dissociation of the disulphide-linked A- and B-chains. The absence of covalent binding to α_2 M/MA and C949Y- α_2 M is thus the result of the absence of ITEs in both α_2 M species. This was exploited to obtain additional evidence for the presence of ITE in WT- α_2 M. WT- α_2 M and p- α_2 M were incubated with 125 I-insulin in the presence of trypsin as in the previous experiments. Again, insulin bound only in a covalent manner with p- α_2 M when trypsin was present (Figure 6). The same result was obtained for the incubation with WT- α_2 M (Figure 6). The binding was not abolished under reducing conditions, indicating that insulin was incorporated in WT- α_2 M via the activated glutamate residue generated during hydrolysis of thiol ester bond.

DISCUSSION

An episomal expression system for α_2 M was developed and used to generate WT- α_2 M and the C949Y mutant found by Poller et al. [6] in a patient with chronic obstructive lung disease. The electrophoretic behaviour of the WT- α_2 M under various circumstances was identical to that of 'slow' p- α_2 M, demonstrating secretion of tetrameric α_2 M with intact ITEs. The electrophoretic behaviour of C949Y- α_2 M was identical with that of methylamine-treated 'fast' α_2 M. C949Y- α_2 M is thus also se-

Figure 5 Cytokine interaction with p- α_2 M, P- α_2 M/MA and C949Y-r α_2 M

(a), (c) and (e) Autoradiographs and Coomassie blue staining of native 5% polyacrylamide gels; (b), (d) and (f), autoradiographs of SDS/polyacrylamide gels 8–16% linear gradient. 'Slow' p- α_2 M (S), 'fast' p- α_2 M/MA (F), C949Y-r α_2 M (M) and samples without α_2 M (—) were allowed to interact with ^{125}I -IL-1 β (a,b), ^{125}I -bFGF (c,d) or ^{125}I -insulin (e,f) as described in the Experimental section. The mobility of 'slow' (S), 'intermediate' (S-1), 'fast' (F) and dimeric (D) α_2 M on native PAGE is indicated. Coomassie Blue staining of gels run under non-denaturing conditions are shown below the autoradiographs of the corresponding lanes. Samples were untreated (—) or treated (+) with SDS or 2-mercaptoethanol (ME) as indicated. All samples in (e) and (f) were treated with trypsin.

creted as a tetramer with a conformation that cannot be distinguished from the wild-type molecule with the four thiol esters hydrolysed. This confirms the observation by Gettins et al. [33], who studied a C949S mutation, that the biosynthesis of an ITE is not needed for the correct folding and secretion of the molecule. However, the presence of ITEs is required for human α_2 M to inhibit proteinases, as C949Y-r α_2 M was not an effective inhibitor of the proteinases examined, in contrast with WT-r α_2 M. We also showed that the mutant competed for binding with the α_2 M/LRP receptor present on human fibroblasts with an affinity similar to that of trypsin- or methylamine-treated WT-r α_2 M or p- α_2 M, demonstrating the presence of a functional receptor-binding domain on C949Y-r α_2 M. The recognition of

C949Y-r α_2 M by the monoclonal antibody F2B2 further confirmed this observation.

The thiol ester mutant allowed us to study the involvement of Cys-949 side chain in 'fast' α_2 M in the interaction of α_2 M with different cytokines [7]. Under native conditions binding of ^{125}I -IL-1 β or ^{125}I -bFGF to p- α_2 M/MA and C949Y-r α_2 M is similar. Marginal binding was observed to 'slow' p- α_2 M. Boiling in SDS reduced significantly the binding to C949Y-r α_2 M but not to p- α_2 M/MA. Under reducing conditions all binding was abolished. This indicates that covalent binding to a p- α_2 M/MA occurs either directly to the Cys-949 through oxidative disulphide bond formation or via a thiol-disulphide exchange mechanism involving a disulphide bond of p- α_2 M/MA with chemical prop-

Figure 6 Identification of ITE in p- α_2 M and WT- α_2 M

SDS/PAGE (8–16% linear gradient gel) analysis of α_2 M allowed to interact with 125 I-insulin was carried out. p- α_2 M (P) and WT- α_2 M (W) were allowed to react with or without trypsin in the presence of 125 I-insulin. Samples were treated with (+) or without (–) 2-mercaptoethanol (ME) as indicated.

erties different from the disulphide bonds of both p- α_2 M and C949Y- α_2 M. Either hypothesis could explain the binding of 125 I-bFGF to the intermediate form of p- α_2 M, where one subunit would show the properties of p- α_2 M/MA.

The much lower covalent binding of both cytokines to C949Y- α_2 M can therefore be explained by a thiol–disulphide exchange mechanism involving other disulphide bonds in α_2 M. This is supported by the observation that this binding is also abolished by reduction with 2-mercaptoethanol.

The significant non-covalent binding of both cytokines to ‘fast’ C949Y- α_2 M but not to ‘slow’ p- α_2 M leads to the hypothesis that the non-covalent binding to ‘fast’ α_2 M might precede the stabilization by either oxidative formation of a disulphide link between Cys-949 and the free thiol groups of the cytokines or a thio–disulphide exchange reaction. In the latter case, the Cys-949 must also be indirectly involved, as covalent binding of IL-1 β and bFGF to C949Y- α_2 M is much lower than to p- α_2 M/MA. The ‘spontaneous’ oxidative formation of disulphide bonds between free thiol groups of bFGF in solution was reported previously by Caccia et al. [17].

A similar stabilization mechanism has also been proposed by Crookston et al. [34,35]. They observed that the non-covalent interaction between α_2 M and transforming growth factor β , which does not contain free thiols [36,37], became stabilized by a thio–disulphide exchange with Cys-949. The velocity with which covalent bonds are formed might depend on the proximity of the free Cys-949 and Cys side chains on the surface of the cytokines. The presence of solvent-accessible free thiols on the cytokine enhances the rate of stabilization.

The situation appears to be completely different in the case of insulin, which does not possess free thiol groups. Covalent incorporation of insulin occurs exclusively when trypsin is added to a mixture of ‘slow’ p- α_2 M with insulin. This binding is resistant to reduction, strongly suggesting that it takes place to the reactive Glx-952 generated on hydrolysis of the ITE, as shown by Chu et al. [32]. The observed covalent incorporation of insulin into WT- α_2 M during trypsin treatment thus further supports the presence of the ITE in WT- α_2 M.

The availability of expression vectors for both WT- α_2 M and C949Y- α_2 M allowed us to mimic the *in vivo* heterozygous situation with a wild-type and a C949Y allele as described by Poller et al. [6] and to analyse the assembly of the α_2 M monomers into a tetrameric molecule. Co-transfection of WT- α_2 M and C949Y- α_2 M into K-562 yielded tetrameric α_2 M with

intact 180 kDa monomers. Electrophoresis in 5% polyacrylamide gels under native conditions generated a pattern with five bands, the upper band migrating with the mobility of ‘slow’ p- α_2 M, and the lower one with the same mobility as ‘fast’ p- α_2 M. This strongly suggests that the intermediate bands are α_2 M molecules with one, two or three subunits lacking an ITE. This is further supported by the observation that, on incubation with the monoclonal antibody F2B2, the intermediate bands as well as the ‘fast’ band disappear and species of lower mobility are generated, indicating the presence of at least one receptor-recognition domain on the α_2 M species with intermediate mobility. This then shows that both the formation of the covalently bound α_2 M dimers and the generation of the tetramers can occur independently of the allelic origin of the α_2 M monomer. However, if a completely random association was to occur then the expected distribution of tetrameric species with respectively zero, one, two, three and four C949Y- α_2 M subunits would be 1:4:6:4:1. In these circumstances almost 94% of the secreted α_2 M would show at least one receptor-recognition domain. Experiments comparing binding of the monomeric rat α_1 inhibitor 3-chymotrypsin complex with the binding of p- α_2 M–trypsin complexes to the cellular α_2 M/LRP receptor demonstrated that high-affinity binding with a K_d of 40 pM occurred when the α_2 M molecule was able to bind two receptor molecules. But even binding to only one receptor molecule resulted in an apparent K_c of 2 nM [38], which is sufficient for the efficient clearance of the species with only one receptor-binding domain from the circulation. At the plasma concentration, the mutation could therefore be considered as a dominant negative. Figure 3 shows, however, that the preponderant species secreted by the WT- α_2 M/C949Y- α_2 M cell line are ‘slow’ α_2 M (four intact thiol esters) and ‘fast’ α_2 M (no intact thiol esters). This indicates that either there is a preferential association of monomers derived from the same allele (or mRNA) or there is segregation of the two episomal plasmids in the transfectant population with some cells carrying exclusively the C949Y- α_2 M cDNA and others carrying exclusively the WT- α_2 M cDNA. This segregation indeed occurs when the cells are kept in culture for a longer period (Figure 4). The observation by Poller et al. [6] of ‘normal’ p- α_2 M levels in the patient heterozygous for the C949Y mutation remains to be explained, however. Indeed, as discussed above, if the monomers associate ‘at random’, only one of 16 tetramers would contain four ITEs and thus 94% of the α_2 M would be cleared from the circulation within minutes of its secretion. If one accepts a preferential association of monomers from the same allele, then this would result in a higher amount of ‘slow’ and ‘fast’ α_2 M than intermediate forms. If the amount of ‘slow’ α_2 M were around 40–50% of the total α_2 M in the blood, then the clearance of the remaining 60–50% would remain undetected in view of the ‘normal’ variation of p- α_2 M levels observed in the population. Further experiments are to be performed to investigate this topic.

This work was supported by the Interuniversity Network for Fundamental Research sponsored by the Belgian government (1991–1996) and by grant 3.0113.94 from the Nationaal Fonds voor Wetenschappelijk Onderzoek. P.M. is a ‘Onderzoeksdirecteur’ of the Nationaal Fonds voor Wetenschappelijk Onderzoek, Belgium. L.v.R. received a scholarship from the Instituut voor Wetenschappelijk Onderzoek in Nijverheid en Landbouw.

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