Inhibition of intracellular proteolytic processing of soluble proproteins by an engineered α_2 -macroglobulin containing a furin recognition sequence in the bait region

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The bait region of the general protease inhibitor α_2 -macroglobulin (α_2 M) was mutated by introducing a recognition sequence of furin. This did not interfere with folding, S-ester formation or tetramerization of the mutant recombinant α_2 M ($r\alpha_2$ M). Mutant $r\alpha_2$ M inhibited furin *in vitro*, by a similar mechanism to that used by plasma α_2 M to inhibit high-molecular-mass proteases. The mutant α_2 M was intracellularly active in COS-1 cells in inhibiting the endogenous processing of the soluble substrates for furin (von Willebrand factor, transforming growth factor β 1 and a soluble form of the envelope glycoprotein

gp160 from HIV-1) but not the membrane-bound form of gp160. The intracellular activity of mutant α_2 M strongly indicated that α_2 M attains its native conformation, and thus that the unusual internal S-ester is formed, before α_2 M passes through the cleavage compartment(s). Our results show for the first time that modulation of the bait region of α_2 M allows the creation of an inhibitor against membrane-bound proteases. It can be expected that the use of α_2 M-bait mutants will become important as a technique for the study of various proteolytic processes and for the identification of the proteases involved.

INTRODUCTION

Human α_2 -macroglobulin (α_2 M) is a homotetrameric glycoprotein present at high concentrations in the bloodstream. The monomeric subunits all contain an internal S-ester (ISE). This unique bond is only found in α -macroglobulins and in the evolutionarily related complement components C3 and C4 [1]. α_2 M inhibits a wide range of proteases by a unique mechanism. Inhibition is initiated by cleavage of a flexible and surface-accessible peptide stretch called the bait region. This cleavage triggers the hydrolysis of the ISEs, followed by a major conformational change. The protease becomes 'trapped' by α_2 M and is thus sterically shielded from its substrate [2]. The bait region sequence is not conserved at all between the known mammalian α -macroglobulin homologues, in contrast with the remainder of the molecule, and the length of the bait region varies considerably between the different α -macroglobulins [3,4]. Although the upper or lower length limit is unknown, Gettins et al. [5] showed that deletion of the major C-terminal part of the bait region prevented tetramerization of human recombinant α_2 M ($r\alpha_2$ M) (see Figure 1). The dimeric mutant $r\alpha_2$ M did not inhibit proteases that cleaved the bait. However, the replacement of seven amino acid residues of the bait by the recognition site of the tobacco etch virus (TEV) protease resulted in tetrameric $r\alpha_2$ M. This mutant $r\alpha_2$ M inhibited the TEV protease as well as other proteases in accordance with the classical α_2 M 'trap' mechanism [6]. To study further the inhibitory potential of bait mutants and structural characteristics of the bait region and their use as reagents for the study of proteolytic processes, we introduced the furin recognition sequence into the bait region of human α_2 M.

Furin is a member of the di- and tetrabasic processing endoprotease family [7]. These enzymes are responsible for the proteolytic maturation of many different proproteins. Furin is synthesized as a 94 kDa proprotease. The propeptide is removed in the endoplasmic reticulum (ER) by autoproteolysis. This is necessary to allow transport of furin to the *trans*-Golgi network, where it is active [8–10]. Furin cycles between the *trans*-Golgi network and the plasma membrane. This trafficking is modulated by the phosphorylation state of a kinase site and by an acidic sequence, both contained within the cytoplasmic domain of furin [11–13]. Its capacity to process many proproteins, together with its intracellular localization and its wide tissue distribution, suggests that it has an important role in the processing of proproteins traversing the constitutive secretory pathway. Although furin is not active in the regulated secretory pathway, most of its family members are. These proteases are sorted together with specific proproteins, mostly hormones and neuropeptides, in secretory granules where processing occurs. The contents of the secretory granules are then secreted from the cell by triggered exocytosis. The sorting mechanism is active at the level of the *trans*-Golgi network in polarized or secretory cells [14–16].

By introducing the furin recognition sequence in the bait region of α_2 M, we expect to gain two kinds of information. First, additional knowledge will be obtained on the physical restraints on a functional bait domain and on the possibility of generating α_2 M-derived inhibitors against large membrane-bound proteases. Secondly, because furin and α_2 M must share the constitutive secretory pathway, we expect to obtain information on the synthesis of active α_2 M and the possibility of using mutant α_2 M as a probe to study protein processing in this secretory pathway.

Abbreviations used: α_2 M, α_2 -macroglobulin; dec-, decanoyl-; DMEM, Dulbecco's modified Eagle's medium; DT, diphtheria toxin; ER, endoplasmic reticulum; FUR/furin- $r\alpha_2$ M, $r\alpha_2$ M produced by K-562 cells that are cotransfected with cDNA species coding for FUR- $r\alpha_2$ M and for furin; FUR- $r\alpha_2$ M, $r\alpha_2$ M containing a recognition sequence of furin in its bait region; ISE, internal S-ester; mAb, monoclonal antibody; p- α_2 M, plasma α_2 M; $r\alpha_2$ M, recombinant α_2 -M; SEC-gp160, soluble form of HIV-1 gp160; TGF, transforming growth factor; TEV, tobacco etch virus; vWF, von Willebrand factor; WT/furin- $r\alpha_2$ M, $r\alpha_2$ M produced by K-562 cells co-transfected with cDNA species coding for WT- $r\alpha_2$ M and for furin; WT- $r\alpha_2$ M, wild-type $r\alpha_2$ M.

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EXPERIMENTAL

Expression constructs for α_2 M, furin and furin substrates

Mutagenesis of the bait region of human α_2 M was performed as described [6]. The primary cleavage site of wild-type (WT) α_2 M [17] was replaced by the furin recognition sequence of von Willebrand factor (vWF) by means of 54 bp oligonucleotide 5'-ATG-GCC-TCT-TCC-CAT-TAC-CAG-TGA-CCG-CTT-AGA-ACG-TAC-ACG-TAG-ACC-TTC-AGG-3'. The α_2 M was designated FUR- α_2 M. WT- and FUR- α_2 M cDNA species were subcloned in pMEP4 and pcDNA3 (Invitrogen). Furin was subcloned from pSVLfurin [18] into pMEP4. The coding sequences of vWF [19] and transforming growth factor β 1 (TGF- β 1) [20] were subcloned in pcDNA3. Constructs containing soluble (SEC) and membrane-bound forms of HIV-1 gp160 and the simian-virus-40 origin of replication were kindly provided by Dr. Weiss and Dr. White [21]. As the expression of gp160 is dependent on the presence of the rev-protein from HIV-1, a construct containing the HIV-1 rev gene and the SV40 origin of replication was obtained from the NIH AIDS Research Reagent Program.

Cell culture and transfection

Human erythroleukaemia cells K-562 were cultured and transfected as described previously [22]. COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco BRL) containing Tes and Hepes (Calbiochem). Cells were seeded at a density of 10^5 per well in 6-well tissue culture plates (Falcon) and transfected 24 h later with Lipofectamine (Gibco BRL), in accordance with the manufacturer's instructions. A total of 3 μ g of DNA was used. The relative amounts of HIV-1 gp160- and rev-constructs were titred to obtain maximal expression of gp160. The amount of α_2 M construct used was 33 % of the total amount of DNA added. The amounts of vWF-, TGF- β 1-, gp160- (WT or SEC), rev- and furin-expression constructs used were 53 %, 53 %, 35 %, 18 % and 14 % respectively of the total amount of DNA added. 'Empty' pcDNA3 was added up to 3 μ g if necessary. The synthetic inhibitor of furin, peptidyl chloromethane RVKR-CMK [decanoyl (dec)-RVKR-CMK] was kindly provided by Dr. Garten.

Western blot analysis

Transiently transfected COS-1 cells were rinsed twice in serum-free medium and transferred to serum-free conditions 30 h after the start of transfection. Stably transfected K-562 cells were washed twice in serum-free medium and seeded at 10^6 cells/ml in serum-free medium supplemented with 3 μ M CdCl₂. When dec-RVKR-CMK was used, it was added to the medium 5 h before cells were transferred to serum-free conditions and also when cells were incubated in serum-free medium at a concentration of 5 μ M. Medium and cells were harvested 15 h later. BSA was added to the conditioned medium (final concentration 5 μ g/ml) and proteins were precipitated with trichloroacetic acid [final concentration 10 % (w/v)]. Cells were lysed in 1 $\times$ SDS/PAGE loading buffer and heated for 10 min at 95 °C. Chromosomal DNA was fragmented by sonication for 15 s. Samples were centrifuged for 10 min at 10 000 g and supernatants were applied to SDS/PAGE gels. On blotting to nitrocellulose membranes, blots were blocked in PBS/0.5 % BSA/0.1 % Tween-20 (PBT) for 1 h at room temperature. Thereafter the membranes were incubated with antibodies directed against furin, furin substrates or α_2 M. Monoclonal antibodies (mAbs) AD3 and ID6, directed against epitopes of gp160 that are located within the first 204

N-terminal amino acid residues (NIH AIDS Research and Reference Reagent Program), were diluted 1:1000 in PBT. The mAbs directed against the catalytic, middle and cytoplasmic domain of furin, designated respectively MON-148, MON-152 and MON-139 [23] were diluted 1:50. A goat antibody against the pro-region of TGF- β 1 (R&D Systems) was diluted 1:1000 and the polyclonal antisera against α_2 M and vWF (DAKO) were diluted 1:2000. Secondary polyclonal antibodies labelled with peroxidase (DAKO) were used at 1:5000 dilution in PBT. Chemiluminescence detection was performed with the ECL detection kit from Amersham.

Densitometric analyses were performed with a Personal Densitometer (Molecular Dynamics).

Analysis of α_2 M

For the production of α_2 M, stably transfected K-562 cells were grown to a density of 8×10^5 cells/ml in DMEM/F12 containing 10 % (v/v) fetal-calf serum, washed twice with DMEM/F12, and seeded at the same density in serum-free medium supplemented with 3 μ M CdCl₂. The conditioned medium was harvested 20 h after the addition of CdCl₂. Purification, treatment with protease and methylamine, electrophoretic analysis, metabolic labelling of transfected K-562 cells and the 'trap' assay with resorufin-labelled casein, were as described [22]. 'Trapping' of furin by FUR- α_2 M was studied with immunopurified human furin. Furin was purified from the membrane fraction of CHO cells over-expressing human furin [24]. Furin was bound to MON-139, coupled to Protein A-Sepharose beads (Pharmacia) in 100 mM Hepes/0.5 % Triton X-100 (pH 6.3). Beads were washed four times in the same buffer and activity towards diphtheria toxin (DT) (Calbiochem) was determined. 'Trapping' of furin by FUR- α_2 M was analysed by preincubating 10 μ l of a slurry of immunopurified furin with 6 μ g of methylamine-treated or untreated WT- α_2 M and FUR- α_2 M for 1 h in incubation buffer (100 mM Hepes/5 mM CaCl₂, pH 7.0). This was followed by the addition of 4 μ g of DT and further incubation at 37 °C for 2 h. Thereafter, the extent of processing was analysed by SDS/PAGE [8–16 % (w/v) gradient gel] (Novex). The amount of furin used, processed more than 50 % of DT within 2 h at 37 °C when assayed in the incubation buffer.

RESULTS

Generation and characterization of FUR- α_2 M

We attempted to generate an inhibitor for furin by replacing most of the primary cleavage site of the α_2 M bait region (residues

Figure 1 Experimentally mutated bait regions of human α_2 M

Primary and secondary cleavage sites within the wild-type bait region of human α_2 M (H α_2 M) are indicated with + [17]. The TEV bait sequence was taken from [6]; GET1 and GET2 represent bait mutants prepared by Gettins et al. [5]. The introduced mutations and deletions are underlined and marked in bold.

Figure 2 SDS/PAGE analysis of $r\alpha_2M$ forms secreted by transfected K-562 cells

Recombinant α_2M in the medium of K-562 cells transfected with one or more constructs encoding WT- α_2M (W), mutant α_2M (F), WT- α_2M and furin (W/f) or mutant α_2M and furin (F/f) (10^6 cells/ml) in the presence of 3 mM $CdCl_2$; overnight labelling with 100 $\mu Ci/ml$ [^{35}S]methionine for (A) was purified by Zn^{2+} -chelation chromatography (A) or enriched by concentration (B, C). Electrophoresis was performed on 7.5% (A) or 6% (B, C) gels under reducing conditions, and Western blotting was performed. The blots were probed with a polyclonal antiserum against human α_2M (B) and with mAbs MON-148 and MON-152 (1:1) against furin (C). (A) is a fluorograph. Detection in (B) and (C) was by chemiluminescence.

Figure 3 Characterization of $r\alpha_2M$ forms secreted by K-562 cells on non-denaturing PAGE

Recombinant α_2M in the medium of K-562 cells, transfected with one or more constructs encoding WT- α_2M (W), mutant α_2M (F), WT- α_2M and furin (W/f) or mutant α_2M and furin (F/f) (10^6 cells/ml) in the presence of 3 mM $CdCl_2$; overnight labelling with 100 $\mu Ci/ml$ [^{35}S]methionine was purified by Zn^{2+} -chelation chromatography. Recombinant α_2M forms were incubated with mAb F2B2 (F_2B_2) or treated with trypsin (TRYP) as indicated. The mobility of native α_2M ('slow') and trypsin-treated α_2M ('fast') is indicated.

683–687) [17] by the furin recognition sequence of vWF (Figure 1). This sequence contains arginine residues at P1 and P4 and a lysine residue at P2 of the furin cleavage site. The introduction of the hexapeptide was such that Arg-681 from the bait region was at the P6 position of the cleavage site. The α_2M cDNA was mutated as described in the Experimental section, cloned into the expression vector pMEP4 and transfected into K-562 cells. The recombinant protein, designated FUR- $r\alpha_2M$, was then purified and analysed together with a WT- $r\alpha_2M$ control. Analysis of both $r\alpha_2M$ forms by SDS/PAGE (Figure 2A, lanes 1 and 2) showed the 180 kDa monomer and the 120 and 60 kDa so-called 'heat fragments', suggestive of the presence of an ISE. With FUR- $r\alpha_2M$, but not with WT- $r\alpha_2M$, significant quantities of 90 kDa fragments were observed. This suggests the proteolytic cleavage of the mutated bait region of FUR- $r\alpha_2M$ by endogenous furin or another proprotein-processing enzyme with a similar specificity.

WT- $r\alpha_2M$ showed a normal 'slow' mobility on 5% non-denaturing PAGE (Figure 3, lane 1). The mobility of FUR- $r\alpha_2M$ was slightly greater in this gel system (Figure 3, lane 2). The combined results from the electrophoretic analysis on SDS/PAGE and non-denaturing PAGE show that FUR- $r\alpha_2M$, as WT- $r\alpha_2M$, is secreted as a tetramer with intact ISEs. However, the conformation of FUR- $r\alpha_2M$ tetramers might be slightly different from the conformation of WT- $r\alpha_2M$.

FUR- $r\alpha_2M$ is a protease inhibitor

One of the hallmarks of α_2M is the inhibition of proteases by a 'trap' mechanism. We therefore investigated whether FUR- $r\alpha_2M$ was still able to inhibit proteases by this mechanism. Resorufin-labelled casein was incubated with elastase or trypsin alone, or with the same proteases, preincubated with purified

Table 1 Inhibition of trypsin and elastase by p- α_2 M and FUR- α_2 M

Resorufin-labelled casein was incubated for 1 h with trypsin and elastase in the presence or absence of p- α_2 M or FUR- α_2 M. The absorbance of trichloroacetic acid-soluble label was then measured at 574 nm. Results are means $\pm$ S.D. for duplicate determinations.

α_2 M form	Enzyme activity ($\Delta A_{574}/h$ per 100 μ mol of proteinase)		
	Trypsin	Elastase	No proteinase
Plasma- α_2 M	0.191 $\pm$ 0.017	0.208 $\pm$ 0.002	0.003 $\pm$ 0.001
FUR- α_2 M	0.192 $\pm$ 0.005	0.207 $\pm$ 0.002	0.006 $\pm$ 0.004
None	0.550 $\pm$ 0.009	0.510 $\pm$ 0.001	0.004 $\pm$ 0.002

Figure 4 FUR- α_2 M inhibits furin by the 'trap' mechanism

WT- α_2 M (W) or FUR- α_2 M (F) (6 μ g) were treated with methylamine (MA) or not treated before incubation with furin (FURIN) for 1 h at 37 °C. DT (4 μ g) was added and incubation was continued at 37 °C for 2 h. The lack of cleavage of DT was obvious only when furin was preincubated with FUR- α_2 M (lane 5). Samples were analysed under reducing conditions by SDS/PAGE [8–16% (w/v) gradient gel]. Lane M, molecular mass markers. The gel was stained with Coomassie Blue R-250.

FUR- α_2 M or plasma α_2 M (p- α_2 M). After incubation, proteins were precipitated and the degradation of casein was determined by measuring the absorbance of the supernatants at 574 nm. The results show that preincubation with p- α_2 M or FUR- α_2 M significantly decreased caseinolysis by trypsin or elastase (Table 1). This indicates that FUR- α_2 M inhibits trypsin and elastase by the 'trap' mechanism.

FUR- α_2 M is able to inhibit furin

We next investigated whether FUR- α_2 M was able to inhibit furin. Two lines of experiments were developed. First, constructs expressing WT- α_2 M or FUR- α_2 M were co-transfected with a furin construct. The corresponding proteins, called WT/furin- α_2 M and FUR/furin- α_2 M, were purified and analysed by SDS/PAGE and 5% non-denaturing PAGE. After SDS/PAGE (Figure 2A), WT- α_2 M could not be distinguished from WT/furin- α_2 M, showing that overexpression of furin does not affect the synthesis and secretion of WT- α_2 M and that the bait region is not cleaved, as could be expected. With FUR/furin- α_2 M, the

90 kDa fragments were now the preponderant species observed on SDS/PAGE. This strongly suggests that overexpression of furin results in the cleavage of the FUR/furin- α_2 M bait region, indicating that the introduction of the furin recognition sequence into the bait was successful. In addition, bands with an apparent molecular mass higher than 200 kDa were observed. This could be the result of covalent bond formation with furin and the reactive glutamic residue that is released by breakage of the ISE. This covalent linkage was documented for the interaction of α_3 M with proteases that contain nucleophiles, mostly lysine residues, on their surface [25,26]. To investigate this, K-562 cells were transfected with the different constructs, the medium was collected, separated by SDS/PAGE, blotted on nitrocellulose membranes and analysed with mAbs directed against cytoplasmic (MON-139) and extracellular epitopes (MON-148 and MON-152) of furin. Furin was detected only in the medium of cells transfected with a plasmid encoding furin, with mAbs MON-148 and MON-152 (Figure 2C). These species showed no reactivity with MON-139 (results not shown), indicating that they were lacking the cytoplasmic domain of furin. The furin detected in the medium is thus most probably the result of proteolytic shedding. After longer exposures, fragments with an apparent molecular mass higher than 200 kDa could be detected with FUR/furin- α_2 M but not with the other transfectants (Figure 2C). Probing of another Western blot carrying the same material, with an α_2 M-specific antiserum, detected the same bands in the FUR/furin- α_2 M preparation (Figure 2B, lane 4). This shows that the high-molecular-mass bands observed with FUR/furin- α_2 M are the result of the covalent linkage of furin to α_2 M fragments.

Analysis of the different α_2 M forms by non-denaturing PAGE showed 'slow' mobility [27] for WT/furin- α_2 M, whereas FUR/furin- α_2 M migrated with a mobility intermediate between 'slow' and trypsin-generated 'fast' α_2 M (Figure 3, lanes 3 and 4). This intermediate mobility was previously observed when p- α_2 M was reacted with high-molecular-mass proteases such as thrombin, kallikrein and plasmin [28–31]. Treatment of FUR/furin- α_2 M with trypsin increased the mobility to 'fast' (Figure 3, lane 12). As a minor fraction of the FUR/furin- α_2 M still migrated as an intact 180 kDa monomer on SDS/PAGE, we investigated whether native α_2 M was still present in the FUR/furin- α_2 M. The mAb F2B2 that specifically recognizes a neoantigenic epitope on the receptor-binding domain of protease- and monoamine-treated α_2 M but not on native α_2 M [32] was used. Incubation of FUR/furin- α_2 M with F2B2 resulted for the whole sample in a slower mobility than native α_2 M, indicating that each tetramer contained at least one subunit with an exposed receptor-binding domain (Figure 3, lane 8). A similar experiment with FUR- α_2 M showed that the mobility of a small part of FUR- α_2 M was also lowered by incubation with F2B2 (Figure 3, lane 6), in agreement with the presence of some bait fragments seen on SDS/PAGE (Figures 2A and 2B, lane 2). The mobility of WT/furin- α_2 M was unchanged by incubation with F2B2. However, treatment of the different α_2 Ms with trypsin, followed by incubation with F2B2, resulted in the expected slower mobility than native α_2 M for all α_2 M forms (Figure 3, lanes 14–17). These results show that the bait region is a substrate for furin, that furin interacts with the fragments generated on hydrolysis of the bait region of FUR- α_2 M, and that cleavage by furin induces a conformational change in the α_2 M, concomitant with the loss of ISEs.

Secondly, we investigated whether FUR- α_2 M can inhibit purified furin *in vitro* from processing a high-molecular-mass substrate. Furin was purified from the membrane fraction of CHO cells overexpressing furin (see the Experimental section). The processing of DT by furin was used as a model system. DT

Figure 5 Interference with endogenous intracellular processing in COS-1 cells by FUR- α_2 M

Conditioned medium (precipitated with trichloroacetic acid) (**A, B, C, D, E, G, H**) or cell lysates (**F**) were analysed by SDS/PAGE: COS-1 cells were transiently transfected with constructs encoding the furin substrates TGF- β 1 (**A, C**), VWF (**B, D**), SEC-gp160 (SEC) (**E, G**) or WT-gp160 (WT) (**F, H**). They were co-transfected with or without constructs encoding WT- α_2 M (W) or FUR- α_2 M (F) in the presence or absence of furin (FURIN) as indicated. The synthetic inhibitor of furin dec-RVKR-CMK (RVKR) was added to the medium of single transfected cells at a final concentration of 5 μ M. Samples were reduced, heated for 15 min at 95 $^{\circ}$ C and analysed by SDS/PAGE (**A**, 8–16% linear gradient gel; **B**, 6% homogeneous gel; **C, D, E, F, G, H**, 6.5–12.5% linear gradient gel). The apparent molecular masses of the proprotein (Pro) and cleaved fragments that are revealed by the antibody used are marked at the right. The apparent molecular masses were estimated by comparison with a molecular mass marker. Recombinant α_2 M present in the medium of COS-1 cells, transfected with a plasmid encoding a particular substrate in the presence or absence of furin or dec-RVKR-CMK, was detected by using an α_2 M-specific polyclonal antiserum (**C, D, G, H**).

is a 60 kDa protein secreted by *Corynebacterium diphtheriae*. After incubation with furin, fragments of 21 and 40 kDa are observed (A and B fragments respectively) [33]. Furin was incubated with FUR- or WT- α_2 M for 1 h, followed by incubation with DT for 2 h. Methylamine-treated α_2 M forms served as negative controls [34]. Normal processing of DT by furin to

the A and B fragments was observed in the presence of untreated WT- α_2 M and methylamine-treated WT- and FUR- α_2 M (Figure 4). Native FUR- α_2 M, however, inhibited the processing completely.

Taken together, the results clearly show that FUR- α_2 M inhibits furin *in vitro* by the classical 'trap' mechanism.

FUR- α_2 M inhibits an endogenous proprotein-processing enzyme intracellularly

We next asked the question of whether FUR- α_2 M, besides its inhibitory action *in vitro*, was able to inhibit proprotein processing intracellularly. The answer to this question would yield information on the folding and 'activation' of α_2 M during its synthesis and secretion. In addition, the use of α_2 M to analyse proteolytic processes could be evaluated.

Therefore COS-1 cells were co-transfected with constructs expressing WT- or FUR- α_2 M and with constructs expressing various substrates of furin: membrane-bound HIV-1 gp160, its secreted mutant form SEC-gp160 and the secreted proteins TGF- β 1 and vWF [35–38]. The efficacy of inhibition by FUR- α_2 M was compared with that of the synthetic furin inhibitor peptidyl-chloromethane dec-RVKR-CMK [39].

After co-transfection of COS-1 cells with constructs expressing TGF- β 1 or vWF respectively and WT- α_2 M, approx. 40 % of the secreted furin substrates were processed from the respective 55 and 330 kDa proproteins to the 44 kDa TGF- β 1 pro-region and to the mature 220 kDa vWF (Figures 5A and 5B, lane 1). In contrast, co-expression with FUR- α_2 M resulted in the complete inhibition of TGF- β 1 and vWF maturation (Figures 5A and 5B, lane 2). This was accompanied by the appearance of 90 kDa bait fragments of FUR- α_2 M (Figures 5C and 5D, lane 2). Dec-RVKR-CMK inhibited the endogenous processing of TGF- β 1 and vWF similarly to FUR- α_2 M (Figures 5A and 5B, lane 5). It also inhibited bait cleavage of FUR- α_2 M significantly (Figure 5C, lane 4). This shows that FUR- α_2 M is able to inhibit proprotein processing of TGF- β 1 and vWF intracellularly, and that the inhibitor is cleaved, most probably in the bait region, by endogenous furin or a furin-like enzyme. Overexpression of furin by co-transfection of a furin cDNA construct completely converted TGF- β 1 and vWF into their mature forms and the co-expressed FUR- α_2 M then consisted of more than 50 % of 90 kDa fragments (Figure 5C, lane 3). These results indicate that the processing capacity of the overexpressed furin exceeds the inhibitory capacity of the concurrently synthesized FUR- α_2 M.

We next studied the processing of a full-length membrane-bound form and a soluble form of gp160. This soluble form, SEC-gp160, is synthesized as a 140 kDa proprotein. After processing, 120 and 20 kDa species are secreted. Co-transfection of plasmids encoding SEC-gp160 and WT- α_2 M resulted in the secretion of unprocessed and mature SEC-gp160. Approx. 50 % of SEC-gp160 was processed (Figure 5E, lane 1). The processing was significantly inhibited when FUR- α_2 M was co-expressed. However, in contrast with the results on TGF- β 1 and vWF, inhibition of the endogenous processing was incomplete (Figure 5E, lane 2). Dec-RVKR-CMK was also unable to inhibit the processing completely (lane 5) but the inhibition was higher than observed for FUR- α_2 M. Overexpression of furin resulted in the secretion of 100 % mature SEC-gp160. Moreover, gp77, which is produced by 'illegitimate cleavage' within the V3 loop, was also detected [40,41]. Cleavage of FUR- α_2 M by the endogenous processing activity and by overexpressed furin was similar to that in the experiments with vWF and TGF- β 1 (Figure 5G).

Membrane-bound gp160 (WT-gp160) is synthesized as a 160 kDa proprotein and processed to 120 and 40 kDa fragments. WT-gp160 was processed by endogenous proteases to approx. 10 % when co-expressed with WT- α_2 M (Figure 5F). The material that migrated just below the 120 kDa fragment was an endogenous protein that cross-reacted with both gp160 mAbs as it was also seen for the negative control (Figure 5F, lane 6). The relative amount of mature gp160 was clearly smaller than seen for the soluble substrates. Co-expression of WT-gp160 with

FUR- α_2 M did not result in a significant inhibition of processing, in contrast with all soluble substrates that were studied. Surprisingly, overexpression of furin did not result in an increased processing of gp160 (Figure 5F, lane 3). The small amount of cleavage by endogenous enzymes or overexpressed furin was not due to a lower overall processing activity present in the cells, as FUR- α_2 M was cleaved to the same extent as in the previous experiments (Figure 5H, lanes 2 and 3). Interestingly, dec-RVKR-CMK was as ineffective as FUR- α_2 M in inhibiting the endogenous processing activity (Figure 5F, lane 5).

DISCUSSION

Transfection of K-562 cells with an episomal construct containing FUR- α_2 M cDNA resulted in the secretion of FUR- α_2 M with the characteristic features of p- α_2 M or WT- α_2 M. FUR- α_2 M is secreted as a tetrameric protein containing intact ISEs. FUR- α_2 M inhibited trypsin and elastase by the classical α_2 M 'trap' mechanism. This shows that the modification of the bait region did not affect the function of the molecule and also that the modified bait region is still a substrate for both proteases.

Our results also show that a limited substitution of acidic for basic amino acids does not affect the functionality of the bait domains. The introduction of the P4 to P2 residues constituting the furin recognition sequence of vWF added three basic residues and eliminated two acidic residues. A more extensive mutagenesis of the α_2 M bait region by which the entire bait region of α_2 M was replaced with that of pregnancy zone protein, merely exchanged the inhibitory spectrum [42]. This further illustrates that the bait region can remain functional after extensive mutagenesis and shows that the bait region defines the spectrum of proteases that are inhibited but does not have an essential role in the secondary and tertiary structure of α_2 M. This is supported by ¹H-NMR experiments indicating that the bait region occurs as a flexible, unstructured and solvent-accessible peptide stretch [43,44] located on a scaffold that is formed by the remainder of the molecule [45].

However, Gettins et al. [46] observed that there are limitations in bait region modification. A decrease in the length of the 39-residue bait region of human α_2 M by 21 and 25 residues, combined with the elimination of two strictly conserved residues at the C-terminal side of the bait (Glu-706 and Tyr-707; Figure 1), did not interfere with synthesis and the formation of covalent dimers. However, no tetramers were formed and the molecule did not 'trap' proteases that cleaved the bait. This study shows that a concomitant change of parameters, such as the length of the bait region and the presence of the conserved residues flanking the bait region, interferes with the formation of a functional inhibitor. This in turn suggests that these parameters might be involved in the correct positioning of the ends of the scaffold, tetramerization and triggering of the conformational change. More studies with mutant α_2 M forms will be needed to dissect the bait region molecularly in order to determine what is required to obtain a functional bait region and a functional α -macroglobulin.

FUR- α_2 M is an inhibitor of furin. Two lines of evidence were developed to prove this. First, co-expression experiments of furin and FUR- α_2 M showed that its bait region is a substrate for furin. In addition we showed covalent linkage of furin and FUR- α_2 M, probably through the reactive Glx-952 residue, after bait cleavage and secretion of α_2 M with an intermediate mobility on 5 % non-denaturing PAGE. It is surprising that the complex of the covalently bound proteins is secreted, as a transmembrane form of furin was overexpressed. This suggests that the covalently bound furin was shed from the cells. However, covalent linkage to α_2 M is not a prerequisite for shedding, as soluble furin was

also found when co-expressed with WT- α_2 M (see Figure 2C, lane 3). Secondly, we showed that preincubation of furin with FUR- α_2 M but not with WT- α_2 M inhibited the processing of DT. Thus the overexpressed furin cleaved the FUR- α_2 M bait region, induced a major conformational change and became covalently incorporated into FUR- α_2 M. Altogether it is clear that FUR- α_2 M inhibits furin with the same characteristics as p- α_2 M inhibits high-molecular-mass proteases.

Furin-cleaved FUR- α_2 M migrated with an 'intermediate' mobility on 5% non-denaturing PAGE. At least one receptor-binding domain was exposed for each tetramer. No 'fast' FUR/furin- α_2 M was observed. Additional treatment with trypsin increased the intermediate mobility to 'fast'. This suggests, by analogy with studies on the interaction of α_2 M with thrombin and plasmin, that one furin molecule did not cleave all the bait regions [28,29,47–49]. It is probably for steric reasons that furin cannot cleave the remaining bait regions and fill every second protease binding site. But the low-molecular-mass protease trypsin was able to cleave the remaining bait regions, known to be located at the bottom of deep cavities [46,50,51]. This was also seen for p- α_2 M, which interacted with plasmin or thrombin. Trypsinization of FUR/furin- α_2 M resulted in a further conformational change.

The successful inhibition of furin *in vitro* led us to investigate the effects of FUR- α_2 M on the endogenous processing of soluble and membrane-bound substrates in COS-1 cells. Inhibition was not really expected because it was unknown whether α_2 M is completely processed to native α_2 M before it travels through the cellular compartments where cleavage of proproteins occurs. Moreover, at the intracellular site where cleavage occurs, conditions are different from those *in vitro*. However, we observed that processing of the soluble substrates was significantly inhibited by co-expression of FUR- α_2 M (Figures 5A, 5B and 5E). Given the introduction of the furin recognition sequence in the bait region, the presence of endogenous furin in COS-1 cells and the localization of furin in the constitutive secretory pathway, the inhibition is very likely to be the result of the interaction of endogenous furin with FUR- α_2 M. Overexpression of furin resulted in nearly complete cleavage of the FUR- α_2 M monomers and in the complete maturation of all soluble substrates tested. The absence of inhibition by FUR- α_2 M in this case must be attributed to saturation of the intracellular inhibitory capacity of FUR- α_2 M by overexpressed furin.

The fact that the inhibition of endogenous processing is observed strongly suggests that α_2 M possesses intracellular protease-inhibiting capacity. The stage at which intracellular inhibition occurs is unclear. Theoretically, inhibition could already occur in the ER. FUR- α_2 M might interfere with the autocatalytic maturation of the enzyme, as documented for furin, and thereby inhibit transport from the ER. This inhibition mechanism would require that α_2 M obtains its native form before it leaves the ER and that it can interfere with autoproteolysis of furin. Although this mechanism is not very likely, it cannot be ruled out. Secondly, inhibition can occur at any stage between the ER and secretion of the substrate from the cell. Again, the requirements for inhibition at this stage are that α_2 M acquires its ISE and tetrameric state when it travels through the compartment where cleavage occurs. The most probable site of bait cleavage, however, is the *trans*-Golgi network as this is the compartment where furin-mediated proprotein processing occurs [7–9]. This implies that the ISE must be synthesized during export through the ER or Golgi compartments. This agrees with the reports that ISE synthesis of the α_2 M homologue rat α I13 and complement component C4 occurred in the ER or Golgi compartments [52,53].

The processing of SEC-gp160 was not completely inhibited by FUR- α_2 M or dec-RVKR-CMK. Processing might therefore be performed by a protease different from furin or possibly in a different compartment from those travelled by FUR- α_2 M. Similarly, the synthetic peptide might not be able to enter the cleavage compartment(s) or show strong inhibition against the responsible enzyme(s).

Only approx. 10% of the membrane-bound form of HIV-1 gp160 was processed by endogenous enzymes in COS-1 cells. This small amount of endogenous maturation was observed before by different authors in different cell lines [54–58]. Most of the uncleaved gp160 might be sorted to the lysosomes or degraded within Golgi cisternae as seen for HIV-1-infected CD4+ lymphocytes or COS-1 cells respectively [54,55]. Surprisingly, processing was not inhibited by the co-expression of FUR- α_2 M or by the addition of dec-RVKR-CMK to the medium. Additionally, co-transfection with a furin construct did not result in a higher processing, as was seen for soluble substrates. As furin is capable of cleaving WT-gp160 [38,41,56,57,59–61], we suggest that furin and gp160 do not share the same cellular secretion pathway in COS-1 cells. gp160 might then be processed by other precursor convertases than furin or by one or more enzyme unrelated to the precursor convertase family, as already suggested by several investigators [59,62–65].

The discrepancy between our results and those of Hallenberger et al. [38] and Vollenweider et al. [41], who showed furin-mediated gp160 processing in African green monkey kidney cells, might be due to the expression system used. Vaccinia virus infection results in the rapid inhibition of host nucleic acid and protein synthesis and affects the fine-tuned mammalian sorting machinery. Together with the high expression levels observed with the vaccinia virus system, this might lead to the 'leakage' of furin into compartments where endogenous furin is never found.

The combined results from this study and from a previous one [6] show that the modulation of the α_2 M bait region leads to proteins with new inhibitory activities against soluble and membrane-bound proteases not inhibited by WT- α_2 M, with widely varying sizes and from different catalytic classes. Bait mutants of α_2 M can therefore be useful tools for the investigation of many proteolytic processes. Their use in cell culture experiments will not be hampered by toxic side-effects. The evolutionary unconserved bait region [3,4], much bigger than that of serpins, might allow the introduction of extended unstructured peptides. Moreover, the covalent linkage of α_2 M to proteases can be exploited to identify and isolate unknown proteases of which only the substrate specificity is known.

We thank Dr. C. D. Weiss and Dr. J. M. White for the gp160 expression constructs and Dr. W. Garten for the furin peptide inhibitor. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pSV-rev from Dr. Marie-Louise Hammarström and Dr. David Rekosh, HIV-1 gp120 mAbs AD3 and ID6 from Dr. Kenneth Ugen and Dr. David Weiner. These investigations have been supported by grant no. 3011394 from the 'Nationaal Fonds voor Wetenschappelijk Onderzoek' of Belgium and by the Interuniversity Network for Fundamental Research sponsored by the Belgian Government (1991–1996). P. M. is an 'Onderzoeksdirecteur' of the 'Fonds voor Wetenschappelijk Onderzoek – Vlaanderen', Belgium. L. V. R. received a scholarship from the 'I.W.T.'.

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